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BIOLOGICAL AND VIROLOGICAL STUDIES OF A NEW SPECIES, <u>ARGAS ARBOREUS</u> (IXODOIDEA, ARGASIDAE), A PARASITE OF WILD BIRDS, WITH SPECIAL REFERENCE TO <u>A. PERSICUS</u> (OKEN, 1818).

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BIOLOGICAL AND VIROLOGICAL STUDIES OF A NEW SPECIES, _ARGAS ARBOREUS_ (IXODOIDEA, ARGASIDAE), A PARASITE OF WILD BIRDS, WITH SPECIAL REFERENCE TO _A. PERSICUS_ (OKEN, 1818)

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I. INTRODUCTION

During an intensive study on the epidemiology of West Nile virus in Egypt, Taylor, Hurlbut and Work isolated a strain of a previously unknown virus from the blood of a 10-year-old child (cf., Work 1962, 1964). The child, a resident of the village of Quaranfil, 15 miles north of Cairo, Egypt, had been suffering from fever and malaise at the time the blood sample was taken.

In their search for an overwintering mechanism of West Nile virus (Taylor *et al*., 1956), several arthropods were investigated and their potentiality to act as vectors and reservoirs was tested. In this effort other strains of the "Quaranfil virus" were isolated from "*Argas persicus*" ticks (Work, 1962). These argasid ticks infested arboreal rookeries of *Bubulcus i. ibis* (common cattle egret) in a few locations near Cairo.

In pilot studies conducted in the NAMRU-3, attempts to isolate viruses from *A. persicus* populations from chicken houses in nearby localities, failed. The question arose as to whether only the *Argas* from heron rookeries was able to harbor and transmit Quaranfil virus, and if so, whether genetic differences between these naturally occurring tick populations were accountable rather than mere chance exposure to the virus.

Genetic differences in susceptibility of vertebrates to viruses are well known. It was suggested by Sabin (1952) that this difference

resided in the cells themselves. The antibody response of the vertebrate host and the degree of tissue susceptibility both seem to play roles which vary in importance depending on the particular situation (Bang and Luttrell, 1961). In invertebrates, genetically based susceptibility - or its converse, resistance - is the greatest single factor influencing the course of development of their parasites (Garnham, 1964). This can be recognized by the fact that usually only a certain proportion of an arthropod population harbors a particular parasite. Studies of the genetic aspects of susceptibility have been made mostly with blood protozoans (Huff, 1940; Ward, 1963), plant viruses (Storey, 1932), and insecticides (Williams, 1951); no comparable work with arboviruses has yet been published. Huff found that when a batch of mosquitoes, _Culex pipiens_, was permitted to feed on a bird infected with _Plasmodium cathemerium_, the causative organism of an avian malaria, only about fifty per cent of the batch developed oocysts, although zygote and ookinete formation were observed in all the specimens. By selective inbreeding of the progeny of resistant and susceptible females for several generations, the infection rate in the former group was lowered to 7.5% and in the latter was increased to 65%. The hereditarily determined susceptibility of this species of mosquito to the parasite was thus demonstrated and was shown to behave as a Mendelian recessive character. In a recent work, Ward (loc. cit.) succeeded in rendering a Malayan strain of _Aedes aegypti_ practically insusceptible to _P. gallinaceum_ after 26 generations of selective breeding. Huff's thesis was confirmed, but a multiple interaction of

genes rather than action of a single gene was suggested.

Storey (1932), in his work with streak disease of maize and the leafhopper vector, Cicadulina mbila, bred races of this insect which were on the one hand able, and on the other hand unable, to transmit the virus in the natural process of feeding on plants. By the crossing of pure races he demonstrated the ability to transmit to be inherited as a simple dominant sex-linked Mendelian factor. In a later work, Storey (1933) demonstrated that non-transmitting races can be rendered infective by parenteral inoculation of the streak virus, "although the numbers of successes were significantly less than by transmitting races." He concluded that in individuals of these races the cells of the intestinal wall resist the passage of the virus, confining it to the gut.

Resistance of arthropods to insecticides, although involving different mechanisms, bears an analogy to the subject under discussion. The occurrence of DDT-resistant populations of flies and lice, and cyanamide-resistant citrus scale insects, are a few examples of many that have been reported.

The fowl tick has been associated with many diseases. The earliest report on the association of A. persicus with disease was given by Traill, 1838a. Hoogstraal (1956) presents a full account of the known disease relations of this tick to man, fowl and cattle. For example, the transmission of the anthrax organism, Bacillus anthracis, to man by the bite of this tick has been reported on one occasion. As a transmitter of the organisms of fowl spirochaetosis, Borrellia gallinarum, fowl piroplasmosis, Aegyptianella pullorum, and

chicken cholera, Pasteurella avicida, it is probably a limiting factor in successful rearing of standard breeds of poultry in some areas (Reid, 1956).

Populations of Argas ticks from heron rookeries have been found infected with Salmonella typhimurium, to which the fowl ticks were negative (Floyd and Hoogstraal, 1956). Wolbachia persica (Suitor and Weiss, 1961) was isolated on a number of occasions from samples of Argas persicus infecting Cairo-area heron rookeries (see also Roshdy, 1961 A, B), but very rarely from those parasitizing nearby domestic chickens (Suitor, 1964). Whether the heron tick plays an active role in the natural infection of young huff-backed herons in the Delta Barrage colony with a piroplasm, Nuttalia sp., is under investigation (Hoogstraal and Garnham, personal communication).

Ticks as purveyors of animal ailments is probably one of the most reviewed subjects. Neitz (1956) published a compendium of the subject, although he did not include human ills. An important regional contribution to aspects of ticks and tick-borne diseases in USSR was filled in by Anastos (1957). Traub (1962) summarized certain phases of the role of ticks in human diseases. In 1963 Philip attempted to cover information acquired since 1956 regarding tick-borne diseases up to that time. An extensive treatment of tick-borne viruses, with special emphasis on the viruses of the tick-borne Russian spring-summer encephalitis complex, was given by Work (1963). The mechanisms of growth and development of animal viruses in ticks were discussed by Rehacek (1965). Kissling from the CDC, is preparing a compilation of

pertinent data on the tick-borne viruses of veterinary importance (1965, in preparation).

In the early phase of this study it was realized that the *Argas* ticks from the heron rookeries were of a distinctive, previously undescribed species. To provide a name to be used in this study and to establish priority for the name, the new species was described and published prior to the completion and submission of this thesis. The abstract of the publication is included here as an integral part of the thesis project.

It should be emphasized that this finding did not alter the goals of the study, that is, to confirm the apparent differences in field infections of the two related tick populations and elucidate the factors involved. The field findings were further supported by comparative infection and transmission experiments.

II. MATERIAL AND METHODS

A. SOURCE OF MATERIAL

The specimens of *Argas* ticks used in this study were collected from several localities. The heron ticks were collected from a heron rookery at Delta Barrage, Qalyub, Qalyubia Governorate, Egypt. The site is at the junction of the two Nile branches forming the Nile Delta, eighteen miles north of Cairo. Here, in a planted forest, a colony of the cattle egret, *Bubulcus ibis ibis*, roosts during the spring, summer and part of the fall each year. The birds support a tremendous population of the ticks. *Casuarina*, *Poinsiana*, *Eucalyptus* and *Ficus* trees are infested with literally inestimable thousands. Large numbers of ticks are usually found in crevices and under the bark, along the stems, to the upper branches where the birds nest. Exuvia and dry specimens intermingled with spider webs are good indications of their presence. Also, scavenger beetles usually are plentiful in an infested crevice.

It seems that in spite of the absence of birds during late fall and winter, other conditions are favorable for the existence and multiplication of these ticks. The site is semi-shaded, the relative humidity high, and the temperature comparatively moderate the year round.

The fowl ticks were collected from chicken houses in three localities: El Mansuriya, Imbaba, Giza Governorate (January, March,

April, May and July, 1963); Saraia el Kobba, Cairo Governorate (June, August, September, October, November, and December, 1963; and January, 1964); and Minshat el Sadir, Cairo Governorate (February, 1964). All localities are populated villages in the vicinity of the City of Cairo. Ticks were collected from the walls of chicken houses which usually are built of sun-dried bricks. Brooders, crevices in wooden fixtures, and spaces under the feeders usually are good sources. Since the widespread use of insecticides, the population of the fowl tick has diminished considerably. For this reason some difficulty has been encountered in collecting the large numbers used for this study.

The ticks were collected in plastic tubes plugged at the bottom with a mixture of sixty parts of plaster of Paris and one part activated powdered charcoal. Patches of muslin held on with rubber bands were used to cover the tubes. At the laboratory, ticks were sorted from all the debris and associated insects in clean Petri dishes. They were then put into clean tubes and labeled with the collection data. To prepare the collections for shipment the plaster of Paris mixture in the tubes was moistened, the tubes wrapped in slightly damp paper toweling and packed in a cylindrical mailing container with a few small holes in the lid. This tube in turn was then put into a larger one which carried the address and a permit obtained from the U. S. Department of Agriculture, Bureau of Enotomology and Plant Quarantine for clearance of the shipment. A dispatch sheet was sent along with the shipment giving the collection data and the observations on the hosts.

Selection of the collecting sites, planning of schedules, and design of methods for periodic collection, sorting and shipping were accomplished prior to embarking upon the thesis project in anticipation of the need for the tick samples.

B. VIRUS ISOLATION PROCEDURE

One of the problems of greatest concern in the processing of field material in the laboratory is to eliminate faulty techniques or conditions which might cause cross-contamination. To solve this problem at CDC a separate room within a laboratory complex was used exclusively for the sorting, identification, grinding, centrifugation and storage of the field specimens. No materials known to be virus-infected were permitted in this room. All virus work on isolates, such as passages and virus identification procedures, was done in another room in the complex. The same precaution was applied to animal rooms in which suckling mice were inoculated and maintained. Primary inoculations (those of field suspensions) were done in rooms separate from "passage" inoculations, which were most likely to be infected with virus and therefore might be a source of cross-contamination.

1. <u>Identification and pooling</u>.

Ticks from each shipment, host, or locality were handled separately. Specimens were dumped from the collecting tubes into a 15 x 60 mm Petri dish half, surrounded with water in a larger dish, 15 x 100 mm in dimensions (fig. 3). This was done as a precaution against the escape of tick larvae which are able to climb up the sides of the glass dish. Adults and nymphs are not able to cling to smooth surfaces. Four 50 ml flasks were set in front of a binocular microscope. Each was fitted tightly with a cone of stiff paper, tip

downward, with an opening provided at the tip large enough to allow a large tick to be dropped through. These cones acted as one-direction traps in case one of the flasks was knocked over accidentally. The flasks were labeled " ♂♂ ", " ♀♀ ", "N_1N_1" and "N_2N_2" (males, females, 1st stage nymphs plus a proportion of small individuals of the 2nd stage nymphs, second and third nymphal instars).

An entomological forceps with angled tip was used to pick up each specimen and place it on a filter paper on the microscope stage. A nonheat-producing American Optical spot-light with a voltage-variac was used as a light source. The identity of each tick was carefully checked while the tick was lightly held down by the forceps tip. The size of the specimen in most instances gave indication of sex and developmental stage. Females are largest, then males, N_2 and N_1 successively. However, the final decision was made by examining the genitalia. The males and females may be readily distinguished by the shape of the genital orifice; that of the male is crescent-shaped while in the female it is merely a transverse slit. The genital aperture in nymphs is lacking or undeveloped; in large nymphs it may be indicated by a small undifferentiated depression. After examination, the identified ticks were placed into the appropriately labeled flasks.

To reduce bacterial contamination and extraneous material on the surface of the ticks, such as dried feces and dust particles, the specimens were washed twice with sterilized buffered water containing antibiotics (see appendix). Enough water to cover the ticks was

added to each flask and the contents swirled periodically over a five minute period for each wash. With the aid of wooden applicator sticks to retain the ticks in the flasks, the water was drained off. The ticks were then transferred from the flasks to Petri dishes, each of which was lined with several sheets of filter paper to serve as blotters. The dishes were covered, labeled with a waxed pencil, and the ticks were left for about one hour to dry.

Since it is not economically feasible or necessary to test each tick separately, they were pooled by sex and stage. The test pool size selected for the heron ticks was five specimens and for the fowl ticks, ten. Factors determining this small pool size were the expectancy of a high rate of infection with the possibility of double or multiple infection (with one or more viruses), and the large size of the specimens.

The number of pools tested for each sex or stage of the heron *Argas* was ten* and that for the fowl *Argas* was five, totaling 40 pools (of five ticks each) from the former and 20 pools (of ten ticks each) from the latter from each monthly collection.

A double set of labels was prepared for each pool using 1/2 inch adhesive tape pulled on a wooden board and a ballpoint pen with waterproof ink. Each label was so coded as to indicate the host, month of collection, sex or stage, and the inclusive numbers of ticks in the pool. For example "Ap-6A ♂ (1-5)" referred to: *A. persicus*, June collection (6) from herons (A); the ticks were males (♂), 1 to 5 in-

*Fifteen for the December, 1963 collection.

clusive. "Ap-3B ♀ (21-30)" indicates A. persicus, March collection (3) from fowl houses (B); these ticks were females (♀) 21 through 30.

The ticks comprising each pool were selected on the basis of equivalent engorgement. This was done mostly by naked eye. Washed ticks were put in sterilized corked Kahn tubes; whenever the maximum pool size was reached, the appropriate labels were stripped off the label board and placed on the tubes. Data on the extent of engorgement of the ticks in each pool were recorded on a work sheet. The tubes were serially numbered, on the tops of their corks, to facilitate later sorting. They were then stored at -65°C in a low-temperature freezer (Revco Chill Chest) until the ticks were further processed for virus testing.

2. Processing ticks for virus isolation tests.

The diluent used in preparing the tick suspensions consisted of 25 per cent normal rabbit serum in 0.05 M phosphate-buffered distilled water and contained 2 mg. of streptomycin sulfate and 1000 units of sodium penicillin G per ml (see appendix for formula). This diluent is commonly used by arbovirologists for virus isolation work because the high proportion of serum provides excellent virus stabilizing properties.

A fresh supply of dilent was made up each week and stored in a refrigerator at 5°C. It was not used when over one week old since the penicillin began to lose its bacteriostatic properties beyond that time. Also, as a precaution against possible viral cross-contamination, separate supplies of diluent were prepared for virus passaging and for

primary isolation tests of field materials.

The tubes containing the tick pools were sorted from the -65°C freezer and aligned in the refrigerator according to the serial numbers on their corks. Five tubes of ticks were removed from the refrigerator at a time for grinding. They were lined up in order in a row on the work table, about nine inches apart; in back of each, a chilled, sterile, two-inch mortar was placed (fig. 4). Two or three drops of diluent were dispensed by pipette into each mortar, followed by a small amount of sterile powdered Alundum (abrasive), applied with a salt shaker. Then, working from left to right, each tube of ticks was dumped into its respective mortar. The corks were replaced and the labeled tubes realigned with the mortars to prevent confusion as to identity. In order, each pool of ticks was ground well in the small amount of diluent to make a smooth paste. Then, with a separate pipette for each pool, 2.0 ml of diluent were delivered to each mortar, and the grinding continued until an even suspension was obtained. Two ml was a convenient volume to use, not being so small as to restrict inoculations nor so large as to unduly dilute the virus in an infected pool. The material from each mortar was then poured back into the respective tube, which was then aligned in a rack in a pan of ice. The procedure was continued until all of the pools were ground, and the tubes placed in order in the iced rack. They were then placed in order in a refrigerated angle-head centrifuge and spun at 3500 rpm (1100 x gravity) for 30 minutes at 5°C. The supernatant fluids were poured off into clean corked Kahn tubes and the labels transferred.

These tubes were replaced in the iced rack and taken to the animal room for inoculation, or were stored at -65°C for later inoculation, if no suckling mice were currently available.

3. Animal inoculation and observation.

Suckling mice were used for the initial isolation of viruses from tick pools. Pregnant female mice were placed in separate mouse cans when they were nearly ready to litter and were checked each morning. The new litters and their mothers were dated and set aside to be used the next day when one day old, or when two days old. Three-day-old mice were not used in this study as it is known that the resistance of mice to some of the arboviruses increases with age. Neither were the suckling mice inoculated on the day of birth, since handling them at this early age increased the likelihood of their being eaten by their mothers.

Litters needed for inoculations were removed from the "maternity ward" and placed in an animal inoculation room. A disposable pliofilm glove was worn on the left hand which held the suckling mice during inoculation, since human odor might stimulate some of the mothers to eat their young. Also, it helped prevent accidental contamination of the operator. Inoculation then proceeded in the following steps: The iced rack of tick suspensions was placed within easy reach. The first mouse can, containing a mother and its litter, was placed on the inoculation table, the lid removed, and all sucklings transferred to a paper towel. One of the two labels was stripped from the first tube and placed on the mouse can. The suckling mice were each inoculated

intracerebrally with 0.02 ml of the tick suspension, using a 1/4 ml tuberculin syringe with a 1/4 inch 27-guage needle (fig. 5). It was important to introduce the needle vertically into the brain case at either side of the sagittal suture to reduce trauma to a minimum and to insure the introduction of the inoculum into the brain tissue. Only six baby mice were inoculated out of each litter and placed back with their mother. These inoculated mice were chosen from among the healthiest of the litter, often with milk showing in their stomachs as evidence of active feeding. The extra baby mice were set aside in a clean mouse can to be added to the litters with less than six babies. After inoculation the can was covered and returned to the shelf, and the one next to it picked up. The order in which the cans of mice were inoculated would be followed in the daily checking for signs of infection. After inoculations were completed, the remaining portions of the tick suspensions were stored at -65°C for future reference.

The test mice were checked each morning for 14 days and the count and symptoms recorded on an "Animal Inoculation Record" sheet, Public Health Service (PHS) form No. 3.494(CDC). For convenience, animal inoculation sheets of field suspensions were kept in a "Primary" book; passages were kept in a "Passage" book.

The standardized abbreviations used were M, missing; E, eaten by mother; D, dead; Pa, paralyzed; Pr, prostrate; Co, convulsing. Additional helpful descriptive designations were Tw, twichy; Wo, wobbly; R, ruffed; and Pa?, questionably paralyzed or with indefinite symptoms, to be observed closely later the same day and on future

checkings. A dash (-) in the checking column indicated normality.

The dead mice and those showing definite signs of infection were saved for passaging as proof of virus infection and for later reference (fig. 6). The sick mice were killed by brief exposure to chloroform fumes in a killing jar, then placed in an end-flap 3"x5" envelope suitably labeled. The label indicated the mouse involved, symptoms, date and page in the record book. Dead and killed mice were stored by date order in the -65°C freezer until processed further.

4. Harvesting mouse brains for passaging.

At weekly intervals, the animal inoculation sheets were inspected and mice selected from the freezer for brain passage in other suckling mice. A serial code number, known as the passage number, was given to each mouse selected and was recorded on the inoculation sheet in the Primary book and also written on the mouse packet with a red pencil. At this time a passage list was made out on a "Passage List Sheet" (PHS form 3.491-CDC), associating the passage numbers with the pages in the primary book where the particular mouse inoculations were recorded. On this passage list sheet, columns also were provided for recording results of bacterial sterility tests on brain suspensions. The passage list sheet was of considerable value in tracking down the laboratory histories of the material tested.

Generally all single, scattered "deads" were passed unless the mice in question were obviously rotten or had died of some known cause. If more than one mouse in a litter died, in a pattern consistent with virus infection, usually only a single mouse of the group

was passed and the remainder held in the freezer until the outcome was known. If the first mouse passage proved negative, a second one was passed provided the symptom or day of death was different from that of the first mouse.

The ultimate goal was to obtain a clear-cut positive or negative result for each tick pool inoculated. Ideally a pool was considered negative if all mice inoculated survived for the full checking period (14 days), or, when dead mice were involved, they were passed with negative results. When one or two mice were found missing on the first day of checking, presumably eaten by their mothers, the test still was considered negative if the others survived for the full 14-day period. If missing mice occurred at later periods, however, a strong possibly existed that they were eaten because they had been sick; in such cases, the original suspensions were reinoculated.

In the passaging procedures it was constantly kept in mind that infected suckling mouse brain contained an enormous amount of highly infectious virus. Therefore, considerable caution was taken to prevent risk of human infection and cross-contamination between different brain specimens. Manipulations were carried out in a properly exhausted hood that had been exposed to U.V. light. After use, the counter of the hood was swabbed with 2% Lysol solution and the U.V. light again turned on.

The envelopes containing frozen mice were arranged in order on one side of the counter according to the passage numbers that had been given. While the mice were being thawed, a matching number of sterile,

corked, Kahn tubes were appropriately double-labeled with the passage numbers. Diluent in a volume of 0.9 ml was then delivered to each tube, and the tubes set in an ice tray under the hood within easy reach.

After the mice were partially thawed, they were dipped in 2% Lysol disinfectant, blotted briefly on a paper towel, and pinned to a soft-wood dissecting board covered with a piece of butcher's paper and a paper towel. One-inch-long round-headed map pins were used to fasten the mouse, one placed through the bridge of the nose and another through the base of the tail. The scalp was swabbed with 1:1000 merthiolate and the swab-stick discarded. With a 1.0 ml disposable syringe and 19 gauge 1" needle, the cranium was pierced and 0.1 ml of the brain tissue sucked up. This was done with ease since the freezing and thawing of the suckling mice caused the brains to lose their texture and become viscous. The respective tube was then picked up, its number double-checked against that on the envelope from which the mouse had come, and the brain material slowly ejected into the diluent. The brain-diluent mixture was drawn up and slowly ejected four or five times until a homogenous suspension was obtained. A bacteriological culture was made by immersing the needle tip into a tube of thioglycollate broth bearing the same passage number.

It should be emphasized that the procedures just described must be executed in a well-ventilated hood, since even when using precautions, a dangerous aerosol may be produced.

The pins were removed and placed in a container of Lysol solution. The mouse carcass and the syringe, with the needle covered with its plastic cap, were wrapped in the butcher's paper and paper towel and placed in a plastic bag for disposal. The dissecting board was covered with a fresh set of papers for the next mouse.

Brain suspensions were centrifuged in the cold (5°C) for 20 minutes at 2500 rpm (1100 G) in an angle-head centrifuge. A separate centrifuge from the one used to spin down the tick suspensions was employed to minimize the chance of accidental contamination of primary material. The supernatants were poured off into sterile Kahn tubes and the labels transferred. These suspensions were then either inoculated immediately via the intracerebral route into suckling mice or were frozen at -65°C to be inoculated later.

Daily mouse checks were made as before. The positive pools were easily recognized, since one or two passages generally resulted in all mice dying within two or three days of each other. The brains of freshly dead, prostrate or paralyzed mice were harvested for identification procedures, using complement fixation, adsorption and neutralization tests. Some were saved to make stocks for future reference.

The brain cultures in thioglycollate broth were incubated in an electric incubator with a thermostatic control set at 37°C. They were read after 24 hours and readings recorded on the passage list sheets. Virus isolations were rarely made from passage material so severely bacterially contaminated as to overwhelm the antibiotics in the harvesting diluent. Similar contamination on a second passage

definitely contraindicated viral infection.

C. PROCEDURES FOR IDENTIFICATION OF VIRAL ISOLATES

1. INTRODUCTION

The arthropod-borne viruses (arboviruses) are the largest family of animal viruses known, with at least 194 currently recognized (Annual Report, 1963, Rockefeller Foundation Virus Laboratory). These viruses are small, most of them ranging from about 30 to 70 millimicrons in diameter (Rivers, 1952) and are ether and sodium desoxycholate sensitive (Theiler, 1957). Their most important characteristic in common, however, is their ability to infect and multiply in both an arthropod vector and a vertebrate host.

Identification of an arbovirus is dependent upon the use of serological tests. The basis for the tests is that when a vertebrate animal is infected with a particular virus, it produces more or less specific antibody against that virus. Some serological tests usually are quite specific; with others, however, there may be a variable degree of overlapping with closely related viruses. Identification of an unknown arbovirus requires the judicious use of both the broad and highly specific tests. The broad (overlapping) tests are used to place the unknown in a "group" within the arbovirus family. Once grouped, the number of specific tests which need to run to obtain final identification is greatly reduced.

Science is indebted to Dr. Jordi Casals of the Rockefeller Foundation (1961) for the classification system of arboviruses currently used based upon the "group" concept. He currently recognizes 21 groups

embracing 146 of the known arboviruses. A remaining 48 viruses still are listed as "ungrouped" (Annual Report, 1963). There must be at least two viruses to form a group; single entities, related to no known group, are listed as "ungrouped" until such time as a relative is discovered and a new group erected.

The most commonly used tests are hemagglutination inhibition (HI), complement fixation (CF), and neutralization (NT), the latter either in tissue culture or in living laboratory animals. In general, the CF and HI tests are least specific, i.e., show greatest overlapping between related viruses. The NT is usually quite narrowly specific.

The first step in identifying an unknown arbovirus isolate is to determine its antigenic group using hyperimmune sera that has been produced against representatives of the viruses in each group. Generally, the four major groups, A, B, C and Bunyamwera, representing 74 of the arboviruses, are checked against first. If the unknown is not one of these, other groups or certain ungrouped viruses are checked against, the selection based upon geographical location, arthropod involved, or other guiding factors. To prepare hyperimmune serum, the immunized animal receives repeated injections of viral antigen. A proper hyperimmune serum has a broad serological spectrum, reactive to a significant extent against all viruses within its particular group. The test selected should be that which gives the greatest overlap. This is generally either the HI or CF test. The HI test is restricted in use to those viruses with hemagglutinating antigens; the CF test, on the other hand, can be used for viruses with or without hemagglutinating

antigens. Infected suckling mouse brain tissue extracts are the usual source for both types of antigens.

The second step, once the group is determined, is to compare the unknown virus with other viruses within its group. For this purpose, a more specific immune serum and the test giving the least cross-reaction are utilized. Antisera with high specificity is prepared by a single virus injection and early bleeding of the immunized animal, when cross-reactivity is weakest.

The usual procedure for identification of an "ungrouped" isolate is to prepare a CF antigen of the isolate and test it against specific immune serum for each of the known ungrouped viruses; the positive findings are then checked by NT (Casals, 1961).

2. PREPARATION OF ANTIGENS

The methods used were those outlined by Clarke and Casals, 1958. Two isolates appearing to be different viruses on the basis of their incubation periods in suckling mice were selected. Both were from heron ticks of the June, 1963, collection. The first, Ap-6A ♀ (11-15) -1SM, passage No. 2481, killed suckling mice on the 8th day. The second Ap-6A ♀ (16-20) -3 SM, passage No. 2482, killed suckling mice on the 3rd day. Two suckling mice of the second passage were selected of each, their brains pooled and given passage Nos. 2558-59 for the first and 2660-61 for the second. These were designated SM_2 brain stocks 2558-59 and 2660-61 respectively, and were stored at $-65^{o}C$ as 10 per cent brain supernatants.

A 10^{-2} dilution of each of the brain stocks was inoculated intracerabrally into 20 litters of 2-day-old suckling mice, 0.02 ml per mouse. These mice were observed closely for signs of disease. When about 10% of the inoculated mice had died following signs of paralysis or prostration, the ones still alive, showing different degrees of symptoms, were killed by quick freezing. The ones which had already died were kept separated and their brains utilized later as a vaccine for producing immune serum.

a. Vacuum harvesting of brains:

The mice were spread out in the hood and left to thaw. Their brains were aspirated directly from the unopened skulls by means of a large 15-gauge needle attached to a vacuum system, as described by Strome (1953) (fig. 7). A flask containing 50% chlorox was used as a safety trap before the vacuum line. Three mice at a time were held on 2" x 2" sterile gauze pads and their brains aspirated; the carcasses were discarded. A 40 ml screw-top bottle immersed in a dry-ice and alcohol bath was used as a receptor.

b. Sucrose-acetone extraction of antigens:

Brain tissues were measured with a 10 ml pipette and hand pipetter and put into a labeled and chilled Stirrer-mixer cup. Four volumes of chilled 8.5% aqueous solution of sucrose were added and the mixture homogenized at 10,000 rpm for one minute. The label was then transferred to a half-gallon bottle. The bottle was fitted with a rubber stopper and placed in an ice bath. Twenty volumes (based on volume of

sucrose homogenate) of chilled acetone ($-20^{o}C$) were put in the bottle. Using a 50 ml syringe with 17 guage, 3-1/2" bevelled needle, the homogenate was slowly ejected, while mechanically stirring, under the surface level of the acetone. After being shaken vigorously, the mixture was left in the ice bath for 10 minutes; then the acetone was aspirated off (fig. 8) and replaced with an equal amount of freshly chilled acetone. After one hour in the ice bath with occasional shaking, this acetone also was aspirated off.

The sediment was poured through a short-stemmed funnel into a 250 ml centrifuge bottle. About 100 ml of cold acetone were used for the transfer. The sediment was then broken to a fine suspension with a sterile Teflon plunger and centrifuged for 5 minutes at 1800 rpm at $0^{o}C$. After the supernatant acetone was removed by aspiration, the sediment was dried under vacuum for two hours (fig. 9).

To the dry powder a volume of chilled saline was added which was 0.4 of the total volume of the original homogenate used. This mixture was refrigerated overnight. The mixture was transferred the next day into 1"x3-1/2" Spinco cellulose centrifuge tubes and sealed with tight metal caps. It was then centrifuged for 1 hour at 10,000 rpm under vacuum in the cold. The supernatant fluid was pipetted off and dispensed into lyophilization ampules in 0.5 ml amounts, using a 2 cc syringe with an egg needle. The sediment was discarded.

After lyophilization of antigen (fig. 10), the ampules were sealed with an oxygen torch. Each ampule was labeled with the isolate designation, stock number, date of lyophilization and amount of

sucrose-acetone antigen contained. They were then stored at $-20^{o}C$ and the box and cell of storage, and number of ampules stored, recorded on "Stock Antigen Record" card (PHS from 300.184 (CDC)).

c. Preparation of 10% seed virus antigen:

To each ml of infected brain tissue, 9.0 ml of 10% monkey serum in antibiotic-treated saline (10% MSS + A, see appendix for formula) were added in a large chilled mortar. The monkey serum was used because it has a high protein content which protects the virus particles by coating them. It should be emphasized that the serum should be normal, i.e., possess no antibodies against the antigen. After grinding to assure a good mix, the suspension was centrifuged at 1500 rpm for 20 minutes in the cold. The supernatant fluid was poured off and dispensed in lyophilization tubes in 0.3 ml amounts. Subsequent steps of lyophilization, labelling, and storing were taken as described above for the sucrose-acetone antigen preparation.

3. PRODUCTION OF IMMUNE SERA

A high purity (99%) beta-propiolactone* (BPL) was used as a viricidal agent in the preparation of killed vaccine. It is a highly toxic material that should never be mouth pipetted. Diluents used were either borate saline, pH 9.3, or 0.2 M phosphate buffered saline, pH 7.4 - 7.8. These are inert, nonproteinateous diluents which do not elicit an immune response when injected into the experimental host.

*Obtainable from "Testagar & Co.", Detroit, Michigan under the trade name, "Betaprone".

a. Preparation of live virus vaccine.

A 10% brain suspension of each stock was prepared in one or the other of the above diluents using the methods of harvesting and homogenizing given under seed virus preparation. Suspensions were centrifuged at 1500 rpm for 15 to 20 minutes at $0^{o}C$. The supernatant constituted the live-virus vaccine.

b. Preparation of BPL-killed virus vaccine.

A 10% brain suspension of each stock was prepared in either diluent and centrifuged as mentioned above. Brain stocks used here were usually from mice that died of infection and were harvested separately from those killed in terminal stages of infection. One volume of 1% BPL in borate saline (pH 9.5) was added (with a syringe) to 19 volumes of the brain suspension. This mixture, constituting the vaccine, was refrigerated for 24 hours, during which time the virus became inactivated and the BPL converted to non-toxic lactic acid.

Both vaccines were stored in appropriately labeled vaccine bottles at $-65^{o}C$.

c. Vaccination of mice for production of hyperimmune sera.

Fifty mice, 4 to 6 weeks old, were inoculated intraperitoneally (i.p.) with 0.2 ml of the killed virus vaccine of each stock (2558-59 and 2560-61) on days 0 and 5. On days 10 and 15 they were inoculated by the same route and amount with live-virus vaccine. Individual mice were anesthetized by brief exposure to ether and bled from the heart on day 24 (fig. 11). Amount drawn did not exceed 0.7 ml of blood per

mouse. Pooled blood was left to stand 2 hrs. at room temperature for clot formation. The clot was then separated from the contained wall with an applicator stick. After refrigeration overnight it was spun at 1000 x G for 20 minutes and the serum decanted into a serum bottle. On day 30 all mice were exanguinated and the serum pooled with that from the 24-day bleeding.

The serum was dispensed in 0.5 ml amounts in Kahn tubes, labeled with the stock numbers, the amount of serum contained and the producing host. These tubes were then stored at $-20^{\circ}C$ and the box and cell of storage and number of tubes stored recorded on "Reference Immune Serum" cards (PHS 3.461 (CDC)). Also indicated on these cards were pertinent data such as the schedule and route of immunization and bleeding, the amount of inoculum, and the type and number of host animals used in the serum preparation.

d. Production of immune and hyperimmune sera in chickens.

Twelve Rhode Island Red chickens, 2-1/2 to 3 weeks old, were inoculated subcutaneously with 0.03 ml each of a 10^{-3} dilution of 2560-61 live stock virus titering $10^{8.0}$ suckling mouse i.c. LD 50 per ml. This amount represented approximately 3000 SM i.c. LD 50 per chick inoculum. Chickens were observed closely for deaths. On day 30 they were bled by cardiac puncture, 5 ml per chicken, for immune serum. Each chicken was then reinoculated both intraperitoneally and intramuscularly with 0.03 ml amounts of 10^{-3} diluted virus. They were bled again on day 45 for "hyperimmune" sera. Sera from the first and second bleedings were so labeled and kept separately. They were stored

and recorded as above.

A similar schedule was followed to produce immune serum against the 2558-59 virus stock. The chickens used were one week old. The virus dilution used was 10^{-2} of live stock titering $10^{7.0}$ suckling mouse i.c. LD 50 per ml. This amount represented about 3000 mouse i.c. LD 50 of virus per chick inoculum.

4. CF SCREEN TEST WITH BROAD GROUP SEROLOGIC ANTISERA

All CF tests were done in accordance with the modified micro-technique of the "Diagnostic Complement Fixation Method (LBCF)."* This method was developed by the Laboratory Branch of the Communicable Disease Center (CDC), Atlanta, Georgia, in 1962 and has been a well-established technique in the general use since.

The sucrose-acetone extracted antigens of prototype stocks 2558-59 and 2560-61 were used after rehydration. Broad group hyperimmune sera** employed in the test were as follows:

1. Broad group A guinea pig serum that had been produced against Mayaro (Tr 4675), Chikungunya, Sindbis, and Eastern Equine Encephalitis (EEE) viruses.

2. Broad group B monkey serum that had been produced in Rhesus monkeys by injection of the viruses of Yellow Fever, Japanese B, West Nile, Dengue II, and Langant.

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*Available in brochure form from the Laboratory Branch, CDC, and will be published (Casey, et al. 1965).

**Prepared by the Arbovirus Lab., Virus and Rickettsia Section, CDC.

3. Broad spectrum Bunyamwera guinea pig serum that had been prepared against Bunyamwera (SM 40-A), Cache Valley (6V-633), Wyeomyia (SM8 H_1) and Guaroa (CDC SM_1 H_1).

Eight serial two-fold dilutions, starting at 1:4 of both the inactivated antisera (30 minutes at 56°C) and the reconstituted antigens were utilized in 0.025 ml amounts in the presence of five 50% hemolytic units of complement (5C'H50)* contained in 0.05 ml. Veronal buffered saline with 0.1% gelatin (VBD) was used as diluent. A serum control (SC) and a normal sucrose acetone antigen control (NSAC) were included for each antiserum in the test to check for nonspecific activities. Three complement controls, in dilutions giving 5.0, 2.5 and 1.25 C'H50 per 0.05 ml, were set with each antigen to check for anticomplementary activity.

After incubation overnight in the refrigerator at 4°C, 0.25 ml amounts of optimally sensitized** 2.8% sheep red blood cells in VBD were added while shaking lightly in a mechanical shaker. Plates used in the microtechnique were then shaken more vigorously, incubated at 37°C for 30 minutes and centrifuged at 600 x G for 5 minutes.

Degree of complement fixation was read, considering all controls were satisfactory (fig. 12). Numerical values were used for recording the tests as follows:

% Fixation	100	70	50	25
Numerical value	4	3	2	1

*Complement obtainable from Probio, Inc., P.O. Box 50, Nyack, N.Y.

**Hemolysin was produced as in Kabat and Mayers.

Values less than 3 were not taken into consideration in the results (figs. 13 and 14).

Broad group C antiserum was not included in this test because it consists of 7 mosquito-borne viruses so far isolated only in the region of Belem, Brazil.

5. CF TEST WITH UNGROUPED VIRUS ANTISERA

In this test three hyperimmune sera prepared against the viruses of Quaranfil (Ar-1113),* Chenuda (Ar-1170), and Nyamanini (AN 2526) were selected. This selection was inferred from the collecting circumstances of the host (the tick) and its geographic distribution. The first two viruses were isolated from *Argas* ticks in Egypt, *A. persicus* (Oken, 1818), from cattle egret rookeries and *A. reflexus hermanni* Audouin, 1827, from pigeon coops, respectively (Taylor, in prep.). The third was isolated from *A. persicus* infesting cattle egret rookeries in South Africa (McIntosh, personal communication). It should also be stated that the Delta Barrage is the topotype locality of Quaranfil virus.

The homologous hyperimmune mouse sera of each of the viral isolate stocks (2558-59) and (2560-61) were also included. The tests were conducted with the appropriate controls, as with the broad serologic group antisera.

*Laboratory designation number.

6. IDENTIFICATION OF THE CRUDE BRAIN ANTIGENS OF TICK ISOLATES

(Figures 13 & 14)

In order to identify serologically the tick isolates in suckling mouse brains, the first step was to check for the ability of the known CF antigens to fix complement with known antisera. The degree of reactivity of each of the antisera was determined for both of the homologous and heterologous antigens. In addition, the end-points of each of the antisera were determined for the homologous antigen. The end-point of a serum is the highest dilution of serum which fixes 5C'H50 in the presence of the optimal antigen dilution.

For this purpose, a box titration micro-CF test was followed. The sucrose-acetone extracted antigens used were isolate stocks 2558-59 and 2560-61, and the viruses of Nyamanini (Ar-1304), Quaranfil (Ar-1095) and Chenuda (Ar-1170). Each of these was tested against its own (homologous) and the other (heterologous) hyperimmune sera. Seven two-fold serial dilutions in VBD starting at 1:8 were utilized in both systems. Test and controls were performed as outlined under 4.

The second step was to substitute the crude brain antigens (unknown tick isolates) for the known antigens and to perform the test using two-fold dilutions of hyperimmune sera starting by two dilutions more concentrated than the end-point of each serum as determined by box titrations.

A simplified technique for producing crude CF antigens from mouse brain isolates was employed. Mice were listed from a virus isolation

sheet; those from the second mouse brain passage and frozen in the prostrate state were preferred. Under the hood, mice were arranged according to list numbers and left to thaw. One row of an equal number of 1/2" x 2-1/2" Spinco cellulose tubes were numbered and arranged in a rack. Two rows of Kahn tubes labeled with corresponding numbers as on the cellulose tubes were arranged behind them in the same rack. The tube rack was then put in an ice tray under the hood.

Using a Caufield pipetter, 1.0 ml amount of chilled borate saline (pH 9.0) were dispensed in the cellulose tubes.

Whole mouse brains were collected using disposable syringes in the same procedure previously mentioned under "harvesting mouse brains for passaging". Any amount in excess of 0.1 ml was put in the Kahn tube on the second row. The 0.1 ml amounts of brains were ejected slowly in the borate saline and mixed thoroughly by drawing and ejecting slowly several times. Spinco tubes were covered progressively with sterile metal caps and the mouse carcasses disposed of in a metal can. Each time after 18 mice were processed, the caps were tightened and tubes arranged serially in an 18-place rotor head in the Spinco ultracentrifuge. Brain suspensions were centrifuged at 10,000 rpm in the cold, under vacuum, for 30 minutes.

The supernatants were then decanted into the respective Kahn tubes in the third row. The procedure was continued until all mice were processed. Tubes containing the excess amounts of mouse brains, in the second row, were stored in the Revco mechanical freezer at -65°C for future reference.

Before tests were performed, 1 ml of VBD (pH 7.2-7.4) was added to each tube of brain suspension to give an approximate 1:2 final dilution of crude antigen of a pH lower than 9.0, since the CF test favors such pH.

A dilution of complement corresponding to seven 50% hemolytic units (7C'H50) was used (Casey et al, 1963). This higher concentration of complement was found necessary to counteract the anticomplementary activity of the brain tissues. Consequently, 7.0, 3.5 and 1.75 C'H50 (0.05 ml) were utilized as dilutions of complement controls. Procedures for the test and controls were followed as outlined above under 4.

7. VIRUS ADSORPTION TESTS IN DUCK EMBRYO MONOLAYER TISSUE CULTURE AND IN SUCKLING MICE

a. Preparation of Pekin duck monolayers for cultivation.

Fourteen-day-old Pekin duck eggs were candled and position of air space and embryo marked. Each egg was then swabbed with alcohol and the shell over the air space removed. The shell membrane was removed next and a sterile glass hook pushed through the allantoic and amniotic membranes. The embryo was hooked around the neck, pulled out, and placed in a sterile Petri dish. Heads, feet and wing tips were removed and discarded. The remaining portions were placed in a sterile beaker and minced finely with sterile 6" scissors. The mince was transferred to a Melnick flask and rinsed with Hank's balanced salt solution (Hank's BSS-X1) to remove excess blood and extraneous membranes. Rinsing was repeated until the solution came off clear. The Hank's BSS

was replaced with 15 ml of 0.25% trypsin, pH 7.4-7.6, per embryo. A sterile Teflon-covered stirring bar was added and the flask placed on a Mag-Mix stirrer base. The digest was kept agitated for 20 minutes at room temperature.

After trypsinization, the dispersed cells were poured through four layers of gauze stretched over the mouth of the Melnick flask into a centrifuge bottle and centrifuged at 600 rpm for 15 minutes. The remaining tissues were covered again with 0.25% trypsin solution and redigested for a second 20-minute interval on the magnetic stirrer. It was again strained and centrifuged; the cells were resuspended in a small amount of growth medium (lactalbumin hydrolysate), pooled with the cells from the first-digestion and then centrifuged in 50 ml graduated conical centrifuge tubes for five minutes to obtain the total packed cell volume. The growth medium was decanted and replaced by a fresh amount sufficient to make a 10% cell suspension. This stock suspension was stored at 4°C until ready for use, up to a maximum of one week. One part of the 10% stock cell suspension was diluted with 20 parts of growth medium immediately before dispensing in sterile 3 oz. prescription bottles in 10 ml amounts with a sterile Cornwall pipetting unit. The bottles were then laid flat in wooden racks, each holding 16 bottles. The racks were shaken slightly to distribute the cell suspension evenly in the bottles. After two days of incubation at 37°C the confluent cultures of healthy cells were selected for inoculation. A pink color of the incorporated phenol red indicator in the medium indicated a favorable pH and a healthy

cell growth. A yellow color indicated acidity and death of cells.

b. Performance of the adsorption test in tissue culture

A total of 42 of the tick pool suspensions judged on the basis of CF to be infected only with virus of the 2560-61 type were tested by the adsorption method for presence of virus of the 2558-59 type as well. Since the pools consisted of 5 ticks each, and more than one of the ticks could have been infected, it was possible that some of the pools may have contained virus of both types. The 2560-61 type, with the shorter incubation period in suckling mice, could have so overgrown the 2558-59 type as to have made it undetectable in the suckling mouse brain CF antigen originally prepared for each isolate.

The adsorption method is essentially a selective neutralization test. Portions of each tick suspension were mixed in equal parts with normal chicken serum and 2560-61 immune chicken serum, incubated, then inoculated into tissue cultures. Suitable controls were run. In the event that both types of virus were present in the tick suspension, the 2560-61 type would be "adsorbed" (neutralized) by the immune serum so that the other could reveal itself.

The tick suspensions were used in full concentration and in 10^{-2} dilution. Controls comprised 10^{-2} through 10^{-6} dilutions of reconstituted seed virus stocks 2558-59 and 2560-61. These were used both alone and mixed in equal parts. The reconstituted seed viruses of Nyamanini (Ar 1304) and Quaranfil (Ar 1055) were also used in the control series in the same dilutions, but only alone and not mixed in equal parts as was the case with the two virus stocks. The diluent

used throughout was 25% NRS + A.

A 0.1 ml amount of each tick suspension was added to a tube containing 0.1 ml of normal chicken serum and to another tube containing a similar amount of 2560-61 chicken serum. One-tenth ml amounts of 2558-59, 2560-61, their 1:1 mixture, Nyamanini and Quaranfil control viruses were added to tubes containing like amounts of normal, 2558-59 immune and 2560-61 immune chicken sera. The 2558-59 and 2560-61 stock mixtures were also added in 0.2 ml amounts to a similar volume of 1:1 mixture of the 2558-59 and 2560-61 immune chicken sera.

The objectives of using this set-up of controls was to determine that every virus was plaque producing and could be neutralized with its own immune serum; also to ensure that there was no loss of titer when a heterologous serum was added. The mixtures of equal parts of the protype stocks was to simulate the mixed infection in the tick suspensions.

The total test consisted of 268 tubes of mixture; these were shaken well to assure good mixing and were incubated in the refrigerator overnight at 4°C to allow the adsorption of viruses by their specific antisera to take place. Then they were ready to be inoculated into tissue culture.

The growth medium was decanted from each bottle of Pekin duck cell monolayers that had been readied for inoculation with the adsorption test mixture. After transferring labels each adsorption test mixture was added to a bottle of cells by pouring in the total amount. When inoculation of each rack of bottles was completed, it

was shaken slightly to obtain an even overlay over the cells, and placed in the incubator for one hour at 35-37°C to allow any free virus to adsorb to the culture cells.

After this one-hour incubation period, each rack was sorted out, bottles arranged in the upright position and their screw caps loosened. Ten ml of warm agar overlay mixture were dispensed in each bottle. The agar mixture was not allowed to touch the mouth or the side of the bottle. After tightening the caps, the bottles were again put in the original horizontal position to permit an even spread of the agar medium over the cell sheet. After 10 to 15 minutes to allow for solidification of agar, the bottles were inverted so that the cell monolayers were uppermost. The racks were then returned to the incubators at 35-37°C. The bottles were observed daily in oblique light for evidence of plaque formation. Numbers of plaques and their types were recorded on PHS form No. 3.325A (CDC).

c. Plaque passaging in suckling mice.

Plaques that were formed after culturing of the adsorbed tick suspensions were passed in suckling mice for identification of their producing viruses. One plaque was pulled at a time with a bevel-tipped sterile Pasteur dropper and ejected in 1 ml of chilled 25% NRS + A in an appropriately double-labelled Kahn tube. A fresh sterile dropper was used for each individual plaque even if it was from the same culture bottle.

The tip of the dropper was aimed only at the chosen plaque without touching other plaques in the cell sheet. Representatives of

every type of plaque were selected for passage from each bottle. All work was done under a properly vented hood. Culture bottles and droppers were disposed of in a discard pan.

Plaque suspensions were then inoculated into suckling mice as described under "passaging of mouse brains". Freshly dead, prostrate or paralyzed mice were saved and brains harvested. The CF method was used for virus identification.

d. Performance of adsorption tests in suckling mice.

The protocol for the adsorption tests in suckling mice was essentially the same as for those just described in tissue culture (Step b). However, the tick suspensions were used in full concentration only. The test controls comprised 10^{-2} through 10^{-6} dilutions of rehydrated seed virus stock 2560-61. Amounts of each dilution were added to tubes containing like amounts of normal and 2560-61 immune chicken sera.

Suckling mice were inoculated with 0.2 ml of each mixture by the i.c. route. Brains of mice that succumbed to virus infection were either used directly for virus identification by the CF test, or were passed into other suckling mice to obtain brain material of the next passage level for such testing.

8. VIRUS NEUTRALIZATION (NT) TEST IN DUCK EMBRYO TISSUE CULTURE

The high degree of immunological specificity of the antigen-antibody reaction in a living system makes the neutralization test especially valuable for verifying results obtained by other less

specific methods. The test was used for two purposes: first, to confirm the diagnosis of some of the virus isolates identified by CF and later proved not to be mixed infections by the adsorption test; and second, to determine the antibody level of each of the immune or hyperimmune sera utilized in the study, expressed in $\log_{10}$ plaque-forming units protection against the virus per ml of antiserum (Log PFU/ML).

Sera: Immune and hyperimmune chicken sera 2558-59 and 2560-61; four immune heron sera; and hyperimmune mouse sera 2558-59, 2560-61, Nyamanini, Quaranfil and Chenuda, were utilized in full concentration. Normal chicken and mouse sera were also included in full concentration as controls.

Viruses: Two isolates (SM brain suspensions), each representing a virus prototype, were selected at random from each of the January, March, August and November tick collection lots for testing. Reconstituted seed virus stocks 2558-59, 2560-61, Nyamanini (Ar 1304), Quaranfil (Ar 1055) and Chenuda (Ar 1170) were used in the control series. Both the test viruses and control viruses were used in 10^{-1} through 10^{-3} dilutions in 25% NRS + A.

The tests were performed in Kahn tubes, properly labeled and held in wire racks. Components of each test were 0.2 ml aliquots each of serum and virus dilution. Serum-virus mixtures, contained in 369 tubes, were shaken, then incubated at $37^{\circ}C$ for one hour. Inoculation of the tissue culture bottles was performed as mentioned under the "Virus Adsorption Test" (7.b) except that the 0.2 ml amount of

inoculum was measured exactly with a pipette and delivered to each bottle instead of merely pouring it off. Tests were observed and readings recorded.

D. VIRUS CHARACTERIZATION

1. Susceptibility of animals

Animals tested for susceptibility to each of prototype virus stocks 2558-59 and 2560-61 included 0.5-day-old chicks, 1-to-2-day-old suckling mice, 3-week-old mice, 4 to 5-week-old chickens, and adult herons. Virus stocks were either utilized in the wet form (a 10% suspension of infected suckling mouse brain from the second passage) or in the lyophilized seed virus form, reconstituted by addition of sterile water before use. Titrations were performed throughout the test by injection of experimental animals or tissue cultures with 10-fold serial dilutions of virus in 25% NRS + A.

Chicks were inoculated subcutaneously with 0.03 ml of each of 10^{-2} through 10^{-8} dilutions of virus. Six chicks were used per dilution. Concurrently, for comparison, the same viruses were titrated by i.c. inoculation of 0.02 ml amounts into suckling mice, and 0.03 ml amounts into 3-week-old mice. One litter, constituting 6 baby mice, and one lot of six 3-week-old mice, were used respectively for each dilution. Surviving chicks were bled for antisera 30 days following incubation.

On the basis of the serial inoculations the LD_{50} titers for each of the virus stocks, expressed in Log_{10}, were estimated by the method of Reed and Muench (1938). Once the titers of the virus stocks were known, desired dosages containing known numbers of LD_{50} units of virus could be calculated for experimental inoculation.

Subsequently, 0.03 ml of a 10^{-2} dilution of each virus was inoculated i.c. and i.p. into chicks, i.p. into 3-week-old mice, s.c. into 4 to 5-week-old chickens and s.c. into adult herons. This was done to compare the effect of the viruses upon these various animals when given by the different routes.

2. Viremia study in baby chicks with each of the prototype viruses.

Wet virus stocks were used. Ten 0.5-day-old chicks were inoculated s.c. with virus 2558-59; another 10 were inoculated with virus 2560-61. In all instances the inoculum was 0.03 ml and contained approximately 3,000 intracerebral suckling mice LD_{50} of the respective virus. To achieve this desired dosage, a 10^{-2} dilution of stock 2558-59 or a 10^{-3} dilution of stock 2560-61 was used. The inoculated chicks were marked with numbered leg bands, placed into two separate brooders at 30°C one for each virus group, and provided with food and water.

The course of viremia was determined by daily bleedings of six chicks from each brooker. When one of the six died, following virus infection or injury, it was replaced by another from the remaining four in the same brooder. The bleedings were continued for ten days after inoculation or until all the chicks had died. Surviving chicks were then bled for antisera one month and two months following inoculation.

In performing the daily bleedings 0.2 ml of blood was drawn from each chick, using a sterile 1/4 ml tuberculin syringe and a 1/4" 27-gauge needle. For more efficient seal between plunger and barrel,

each syringe was moistened with sterile physiological saline before use. The blood was ejected into 0.9 ml of chilled 25% NRS + A and mixed gently. A soft clot formed, which was loosened from the side of the tube by flicking several times. After centrifugation in the cold at 1500 rpm for 15 minutes, which threw down the red cells, the supernatant constituted an approximate 10% (10^{-1}) dilution of chick serum. The LD_{50} titers of the daily blood samples from the chicks (viremia levels) were determined by first screening in suckling mice; i.e., inoculating the 10^{-1} dilution to eliminate those samples containing very little or no detectable viruses. Those found positive were then titrated (serial 10-fold dilutions were inoculated) to determine the LD_{50} endpoints.

During the viremia study with virus stock 2560-61, fresh feces from chicks showing gastrointestinal troubles, manifested by diarrhea, presumably from virus infection, were diluted, screened, and titrated to determine the presence and concentration of virus in each case.

3. Viremia study in adult herons with the prototype virus stock 2560-61.

Two cattle egrets, *Bubulcus i. ibis*, and two snowy egrets, *Leucophoyx t. thula*, one year of age, were used in this study. The birds were provided when only about 3 to 4 weeks of age by Dr. William Jennings, Florida State Board of Health, who collected them from the Bush Key rookery in the Tampa Bay area of Florida in July, 1963. They were maintained in captivity at the Communicable Disease Center on chopped mullet and baby mice until mature. The test followed the

protocol previously outlined for the viremia study in chicks. Daily bleedings were made from the wing veins or the jugular vein. The one-month and two-month bleedings were made from the heart.

4. Behavior of the prototype virus stocks in tissue culture.

Studies on the behavior of the 2558-59 and 2560-61 virus types were conducted in the duck embryo monolayer tissue culture system as part of the controls for the neutralization tests in tissue culture. Each stock virus was tested for the kind of cytopathic effect it produced, plaque formation, and type of plaques formed.

5. Hemagglutinating activity of the two virus prototypes:

Since the discovery by Hirsh in 1941 that influenza virus will agglutinate the erythrocytes of some species of animals, and that this agglutination could be inhibited by specific antiviral serum, this test has been utilized as a diagnostic technique (Burnet, 1952). Different workers have showed that those arboviruses with hemagglutinating activity had rather specific requirements as to pH and type of erythrocyte (Clarke and Casals, 1958).

For the purpose of this study the Clarke and Casals (1958) technique was followed using a microtiter system adopted by CDC. Aliquots of each of the sucrose acetone antigens (see Section C) were reconstituted and diluted 1:10 in chilled, 0.4% bovalbumin-borate saline (BABS), pH 9.0, and placed in the refrigerator for one hour. Eight two-fold dilutions of each antigen were prepared in 0.05 ml amounts in borate saline (pH 9.0) by serial dilution with .05 ml loops

in micro-plate wells. An equal volume of stock goose-red cell suspension diluted 1:24 in the appropriate adjusting diluent to give pH's 5.7, 6.0, 6.2, 6.4, 6.6 and 6.8 was added to each well using a different micropipet for each pH concentration. Plates were then shaken, incubated at 37°C for 1 hour and then read for the pattern of hemagglutination.

The stock goose cell suspension used here was of 1:40 dilution in dextrose-gelatin-veronal (DGV), that had been standardized to give an optical density of 0.450 as determined in a Coleman Junior Spectrophotometer operating at a wave length of 490 mu. and using cuvettes of 10 mm. internal diameter.

E. THE LABORATORY REARING OF *ARGAS* TICKS

In rearing ticks one of the first considerations was to eliminate the danger of infestation of the premises with the parasite. This required care in manipulation and complete isolation. Before any attempt to rear the ticks was made, it was essential that certain facilities and items of equipment were available. A small room was set aside and used solely for the purpose of tick rearing and storage. An inside room was used so that the interior was never exposed to direct rays of sun and not subject to extremes of temperature. The temperature variation was between 18°C and 27°C and proved satisfactory. The use in the tick room of paint containing residual insecticides or fly sprays which may contaminate the equipment was strictly avoided.

1. Equipment:

Two moated tables 7' long by 2'10" wide by 30" high were constructed (fig. 15). One was used for placement of four stainless steel "rabbit" cages, in which pigeons were kept; the second was used to place rearing shelves, jars, cloth bags and other items of equipment used in handling the ticks. A third 3' x 2' moated table, on rollers was utilized as a movable work table and was furnished with a binocular microscope. All tables were made of galvanized steel with a moat 1-1/2" wide by 1-1/2" deep all around the edge. It was intended to fill the moats with mineral oil as a barrier against escape of ticks but it was found that smearing the inside rim of each moat

with petroleum jelly was just as satisfactory and much less messy.

A 3-shelf electric incubator with thermostatic controls set at 27°C provided an even temperature for tick incubation. On the bottom shelf a number of open finger bowls containing water were placed to maintain a relatively high humidity inside the incubator. A drum type hygro-thermograph was employed to adjust the relative humidity to 75% by adding or removing finger bowls until this desired point was reached.

Cellulose nitrate transparent tubes of two sizes, 25 x 100 mm and 13 x 100 mm, were prepared for use in holding ticks by cutting a circle off the bottom of each just below the curvature with a sharp scalpel. Then, as quickly as possible, the tubes were planted about 3/4" deep in an enamel tray half filled with a watery paste consisting of one part of activated charcoal powder and 60 parts of plaster of Paris thoroughly mixed with water. After the plaster hardened, the tubes were twisted loose; each now contained a plug of the charcoal-plaster at the bottom. This material was beneficial in absorbing the tick secretions and eliminating fungus growth. Also, it could be moistened with water if necessary to raise the humidity. Patches of muslin held on with rubber bands were used to cover the tubes and prevent ticks from escaping. Contaminated tubes were cleaned with flowing tap water and a bottle brush and sterilized in a safety hood by exposure to a UV light for at least 24 hours. No disinfectants were used since they might remain on the plastic and be injurious to the ticks.

A type of tick-retaining screw-capped capsule was devised for feeding ticks on pigeons (fig. 16). The capsules had an inside diameter of 15 mm and height of 17 mm, and were cut from square-shouldered, plastic-capped polyethylene squirt bottles. A finished capsule consisted of the threaded neck of the bottle, with a collar around its base 5 mm in width, and the screw cap. The collars had to be thinned by a lathe to give them more flexibility and a better fit against the skin of a pigeon or chicken. A circle 15 mm in diameter was cut in the top of each screw cap and replaced with plastic screening, 32 mesh per inch. This screening permitted viewing; it also allowed ventilation and thus prevented condensation of perspiratory fluids. Polyurethane foam pads, 40 mm x 35 mm, were cut from sheets 5 mm thick. A hole was cut in the center of each pad with a cork borer 15 mm in diameter. These pads were cemented to the capsule collars with "Duco" plastic cement and served to make a tight seal against the skin of the bird and prevent skin irritation. The pads were used only once and then discarded. To prevent delays during the course of an experiment, an extra supply of clean capsules fitted with pads was kept on hand. Capsules were cleaned in the same manner as the tick rearing tubes.

The capsules were found to be very satisfactory in rearing and controlling the large numbers of ticks needed for transmission studies by providing for full recovery of the original numbers of ticks placed on the host and preventing the host from injuring them.

Other items of equipment included entomological forceps, fine watchmakers forceps, fine camel hair brushes, a supply of 1-1/2" adhesive cloth tape, a pair of bandage scissors, a wooden board, 2"x2" gauze pads, and two squirt bottles, one filled with 70% alcohol and the other with physiological saline. In addition to these items there were several large glass jars 1' in diameter fitted with lids of 1/4" mesh hardware cloth, and cloth bags with elastic openings wide enough to fit snugly around these jars; 400 ml beakers, cheese cloth, and heavy black cloth. These last items were used in feeding ticks on herons, large chickens, and baby chicks when not using the tick feeding capsule.

2. Methods of feeding ticks:

Two methods of feeding ticks were utilized, by use of the capsule described above, and in the large jars. With the capsule method, young adult pigeons were used as hosts because of their comparative neatness, favorable size and tolerance toward handling. Each pigeon was prepared by crossing its wings, lightly binding its legs with a rubber band and plucking the feathers in an area approximately 1-3/4" wide around the body, beginning anteriorly in an imaginary triangle extending from the two wing joints to the tip of the sternum. Plucking was done along the direction of feathers without tearing the skin. The area was then rubbed with a gauze pad soaked with 70% alcohol to remove grease and scales. Strips of the 1-1/2" adhesive tape 10" long were spread on the wooden board. With a cork borer a circle of 17 mm in diameter was cut in each, about 1" from the end. On the other end

a longitudinal slit 10 cm long was made with a scalpel. The tape was then stripped off the board and the neck of a padded capsule inserted into the circular opening in the tape from the adhesive side. The capsule was placed on the area rich with blood capillaries in the axillary region and the tape wrapped firmly, but not tightly, around over the keel, the axillary region of the other side, and the dorsal region. Where the tape met again the two end strips were separated to encircle the capsule. After inserting the ticks to be fed inside the capsule, the cap was screwed into place. Numbered aluminum leg bands were used to mark the pigeons and an experimental host record was kept. After freeing the pigeons in their cages, they staggered for a few minutes but soon became accustomed to the tape and behaved normally.

Feeding the larvae presented a problem because of their minute size and active movement, making transfer to the capsule difficult. This problem was solved by putting a few drops of physiological saline inside the cell after it was attached to the host and transferring the larvae to this wet surface with a wet camel's hair brush. The moisture held them so they could not escape. After replacing the screw cap the fluid was drained through the top mesh. A few touches with the brush to the mesh absorbed most of that remaining. In a short time the larvae were completely dry. No restraint of the bird was needed after the ticks were secured in the capsule.

Because of the negative geotropic behavior of the heron _Argas_ ticks, it was possible to feed as many as 20 adults, an even greater

number of nymphs or larvae, at a time in one capsule. Ticks that had completed feeding climbed up from the skin surface to the screw cap and left room for others to feed. Daily examinations of feeding ticks were done by flicking the capsule and removing the cap or by looking through the top mesh with the aid of a flashlight. Capsules were kept on pigeons for periods up to 2 weeks until feathers started to grow again.

The second method of feeding ticks was used in the early stages of this study and later for the transmission studies. Larvae were put with the hosts in appropriate sized containers, 400 ml beakers for 1/2 day old chicks and the large glass jars for other chickens and herons. When using young chicks, a layer of coarse wood shavings was put in each beaker to absorb the excretions of the chick, to release its weight off the engorged ticks, and to act as a hide-away for the ticks. Since larvae can climb up smooth surfaces (contrary to adults and nymphs), the inside rim of each beaker was painted with petroleum gel. The beakers were then covered with patches of cheese cloth held on with thick rubber bands, put into enamel pans surrounded with water and detergent, and placed in the dark in the insectary or under a dark cloth. This procedure was done as quickly as possible lest the ticks be picked up and eaten by the chicks. It was advisable to put the ticks into the prepared containers first, and then, in the dark, distribute the tagged chicks and place on the cheesecloth covers, using a flashlight shielded with a piece of red cellophane.

After allowing 24 hours for attachment of larvae, the chicks were removed from their beakers and placed in a corresponding number of the large jars. The beakers were discarded after 15 minute exposure to chloroform vapor. Each jar was inverted over its mesh lid and fitted with a cloth bag for the larvae to drop into after completion of engorgement. A tick-rearing shelf was used to support the jars over matching holes and allow the cloth bags to dangle through. The chicks were fed dampened chick mash in small beakers. The bags were removed and examined daily for larvae that had completed feeding. They were immediately replaced with clean collecting bags.

When using large birds for hosts, practically the same method as for the young chicks was followed during the attachment period, except that a base of metal mesh had to be used inside the containers over the wood shavings. Also, heavy metal lids had to be placed on top of the cheese cloth covers to keep the birds from escaping.

Feeding of the adult and nymphal argasids was much simpler than feeding the larvae. There was no need to use petroleum jelly or water pans as barriers since these stages could not climb up the glass surfaces. Also, since they usually fed and detached in less than one hour, cloth catching bags were not utilized. It was necessary only to search the wood shavings for engorged ticks.

3. Maintaining ticks apart from the host:

Tubes of ticks were arranged in metal trays 4" wide by 25" long by 3-1/2" deep half-filled with sand. When the trays were kept in an open room, the sand was moistened to maintain a favorable humidity

within the tick rearing tubes. Moistening was not necessary when the trays were kept inside a humidified incubator. In all cases excess moisture was avoided.

Inspection of the whole stock of ticks under study was made daily as a matter of routine.

4. Keeping records:

It was imperative to keep meticulous records relating to the ticks under observation. A card was kept for each pair of ticks (male and female); the designation number given was that of the female. If the male died, it was replaced with another male; however, if the female died the male was discarded. The progeny of a female were given abbreviated descriptive titles, including the mother's designation number, their descent level and their own designation number. For example, the title Ap-A ♀ 6-3f_1-N_2(1-6) stands for *A. persicus* (Ap) from herons (A), ♀ No. 6's third egg batch laid (3), first filial generation (f_1), second stage nymphs designated 1 through 6, pooled together.

A different card was kept for each batch of ticks that showed a different parasitic period. Records of egg counts, moltings and feedings, as well as observations of the behaviour of sexes either on or off the host, such as copulation or emission of coxal fluids, were kept on the particular cards. In tabulating results, in place of dates entered in the original cards, the number of days which have elapsed was utilized. This was to avoid the confusion which would result if the length of the parasitic period had to be calculated in

every case from the original dates.

F. EXPERIMENTAL INFECTION OF *ARGAS* TICKS WITH THE PROTOTYPE VIRUS STOCK 2560-61 AND THE VIRUS OF ST. LOUIS ENCEPHALITIS

1. Introduction

These experiments were designed to accomplish two main goals. The first was to determine the infectivity and course of infection of the prototype virus stock 2560-61 in the apparent natural host, the cattle egret, and in an experimental host, the baby chick. The second goal was to demonstrate the capability of the heron *Argas* to transmit this virus by bite and to compare its vector efficiency in the laboratory with that of the fowl *Argas*.

To permit a broader view of tick-virus relationships, a mosquito-borne virus was included in the study; attempts were made to infect the heron ticks with a Texas strain of St. Louis encephalitis virus.

Several quantitative methods can be used in the laboratory to estimate vector efficiency and to compare that of one vector candidate with another. Foremost among these are the threshold of infection, the infection rate, and the transmission rate (Chamberlain, *et al*, 1954).

The threshold of infection is defined as the minimum concentration of virus capable of causing an infection of a certain proportion of the individual arthropods ingesting it. Usually this proportion is set at 1 to 5 per cent; a 50 per cent infection level is also a useful determination. The lower the infection threshold of an arthropod species, the higher its vector potential rating since a species of

such susceptibility presumably would have a better chance of becoming infected in nature.

The infection rate is the percentage of specimens becoming infected (having virus become established in their bodies) when fed a meal containing an optimal concentration of virus, i.e., the highest concentration generally attained in the blood of the preferred vertebrate host. For significant comparison, each species tested should receive meals containing virus in equivalent concentration.

The transmission rate is the percentage of arthropods tested which become capable, after an appropriate incubation period, of transmitting by bite following the optimal virus meal. It is an even more critical measure of vector efficiency than the infection rate.

In ticks (and mites) transovarial passage of viruses from an infected female to her progeny also indicates a high vector potential; it suggests a long phylogenetic association between the tick and the viral "parasite." Transtadial transmission, on the other hand, appears to be of lesser significance in rating a vector. Since the stomach with its diverticula, which contain the infected meal, is among the organs that do not undergo fundamental reconstruction during ecdysis, presence of virus in a succeeding stage is not necessarily a unique phenomenon (Balashov 1963, in Raechek, 1965).

2. Establishing "clean" colonies of heron ticks.

In view of the fact that transovarial transmission of viruses is theoretically possible in ticks (Reeves, 1958), the importance of using laboratory-reared ticks of a known "clean" (non-infected) line is

readily understood. In this study, the progeny of each pair of ticks were kept separately. By tagging a pigeon to be used only for each pair and their progeny, intermixing between hosts was prevented. As a further precaution, each pigeon was kept in a separate cage. When enough f_1 adults from one pair of parents accumulated, a proportion (approximately one-third) was ground individually and inoculated into suckling mice to prove lack of transovarially acquired infection. The remaining two-thirds were then assumed to be uninfected also and were reared further to establish clean colonies. Each colony was given the designation number of the female grandparent.

3. Transmission study with virus stock 2560-61 in heron Argas and fowl Argas ticks.

Half-day-old chicks were inoculated subcutaneously with 0.03 ml of a 10^{-3} dilution of the 2560-61 virus stock, representing approximately 3000 intracerebral $SMLD_{50}$ units of the virus. The chicks were marked with numbered leg bands, placed in a brooder at 30^{o}C and watched closely for symptoms (fig. 17). As soon as a few were observed to have died after short periods of paralysis or prostration, the survivors were bled (0.2 ml in 0.9 ml of NRS + A) for later titration to determine viremia levels; immediately after the blood samples were taken, normal (clean) ticks were placed upon these chicks so that they could obtain an infected blood meal. Both the fowl *Argas* and heron *Argas* ticks were used, but upon different infected chicks. The feedings were accomplished in the insectary in the manner described under tick feeding techniques. Either five males and five females, or ten

nymphs were left with each of the infected chicks overnight (fig. 18). Those adult ticks that fed on a chick were given a serial transmission number (Tr. No.) and paired, 1 ♂ with 1 ♀, in an appropriately labelled rearing tube. Odd single fed males or females were paired with single clean males or females. The nymphs were kept singly and labelled accordingly. Part of the fowl ticks that fed on each of four chicks were kept in mass, and given a single Tr. No.

After the tick feedings, the surviving chicks were bled again. Each of those which had died during the night, apparently from infection, was dissected under a safety hood and the heart transferred aseptically to a sterile 15 x 60 mm Petri dish. The heart was then sliced into 2 or 4 pieces and the serum from the heart blood clot allowed to drain out. One-tenth ml of this serum was drawn up with a 1 ml disposable syringe and mixed with 0.9 ml of 10% NRS + A diluent. The 10% dilutions of sera from the pre-tick feeding and post-tick feeding bleedings and the heart clots were titrated in suckling mice to determine the viremia range of each chick during the tick feeding period.

The ticks were incubated in the insectory at 27°C and 75% relative humidity. After about 20 days, they were allowed to refeed on normal 0.5-day-old test chicks. This was done by marking each chick with a leg number then exposing it overnight to the bites of a pair of "infected adults" or a single "infected" nymph. Each tick was examined carefully after the transmission feeding period to determine whether or not it had actually taken a blood meal. Those chicks not fed upon

served as controls for the test. All chicks were placed in a brooder at 30°C and provided with food and water.

Starting the 4th day (the shortest incubation period as determined by viremia studies), chicks were checked for signs of infection every four hours, at 8 and 12 A.M. and 4 and 8 P.M. Those showing symptoms were bled (0.2 ml in 0.9 ml of diluent) to screen for virus as evidence of transmission by tick bite. Their brains were also taken for passage in suckling mice. Brain tissue and heart-clot sera were taken from chicks found dead. All chicks which survived without symptoms until the sixth day were bled at that time and tested for viremia also, since it was possible that inapparent infections might have occurred. These chicks were held and bled again on the 30th day. Whole sera obtained from the 30-day bleedings were tested for neutralizing antibodies against the 2560-61 virus strain in duck embryo monolayer tissue cultures. The neutralizing antibody level was determined for each serum expressed in Log PFU/ML protection against the virus.

Similar attempts to refeed the ticks were made every two or three weeks to obtain as accurate measure as possible of the extrinsic incubation period of the virus.

After every feeding, eggs laid by "infected" females or by clean females previously paired with "infected" males were left to hatch. The offspring (larvae) were given the transmission number designation of their mothers and the descendant level number (i.e., $2f_1LL$ for larvae produced from the second oviposition). Whole batches of larvae were ground together in 1 ml of diluent and inoculated into suckling mice

in attempts to detect virus as evidence of transovarial or transspermatophoral transmission.

Nymphs that molted to adults or nymphs of the next stage after the initial infective feeding were tested singly for evidence of transtadial transmission. Part of those which had molted to females were paired, each with a clean male, fed on normal chicks, and resulting eggs left to hatch. The adults were then screened, the chicks tested for infection, and the larvae obtained also tested for virus. By this procedure, all three types of transmission were investigated in the same ticks, transtadial, bite, and transovarial.

At different intervals of extrinsic incubation a small amount of coxal fluid was extracted from each tick for virus testing. Under a dissecting microscope each tick was held, ventral side up on a clean filter paper, with sterile forceps. The area just above the genitalia and between the first coxae was teased with the tip of a sterile capillary 0.7 mm in diameter. The coxal fluid usually formed as a spherical drop at either opening of the two coxal glands. The fluid was then drawn up by capillary action and blown gently into 1 ml of chilled diluent. After mixing by gentle shaking, it was inoculated into suckling mice to test for virus.

At the end of the transmission study, each tick was ground in 1 ml of diluent, centrifuged, and the supernatant (considered as 10^0 or undiluted material) inoculated intracerebrally into suckling mice to test qualitatively for presence of virus. Some of the most heavily infected tick suspensions, judged by their killing all of the suckling

mice inoculated (6/6), were given serial ten-fold dilutions and reinoculated into suckling mice to obtain an estimation of the maximum amount of virus contained per tick. Since all of the virus contained per tick was initially suspended in 1 ml of diluent, and 0.02 ml amounts were inoculated per mouse, each mouse received one-fiftieth of the total virus, or 10-fold dilutions thereof. Therefore, the titer of the virus suspension, plus the dilution factor of 50, or 1.7 logs, provided a reasonable estimation of the actual amount of virus, in LD_{50} units, contained per whole tick. Estimations of the virus titers in the ticks that killed only 3/6 or less of the mice inoculated were based upon the single litter inoculated in the screening test, without further serial dilution and inoculation.

The results of the initial tick screenings and the selected titrations were used as bases for calculating the infection rate and drawing conclusions as to virus persistance and multiplication.

4. Transmission study with the virus of St. Louis encephalitis in the heron *Argas* ticks.

Chicks 0.5 day old were inoculated via the intraperitoneal route with 0.03 ml of such dilution of virus as to provide approximately 10,000 SM LD_{50}. The virus strain used was Texas 16017 M_2SM_2 (4/9/63). SLE virus causes only subclinical infections in chicks, but reliably produces a viremia in the chicks which reaches a peak from 48 to 72 hours of incubation (Sudia and Chamberlain, 1959). The chicks serving as virus source for tick infection were used at 48 hours of virus incubation and exposed to ticks overnight. Blood samples, 0.2 ml in

0.9 ml of diluent, were taken from each chick immediately before and after the period of tick feeding exposure. These were titrated in suckling mice to estimate the titer of virus in the blood ingested by the ticks. In subsequent attempts to demonstrate transmission of the SLE virus to normal chicks by the bite of these ticks, the chicks were bled also 48 to 60 hours after the exposure to ticks, this being an optimum period for detection of viremia as evidence of virus transmission by tick bite.

Transovarial and transtadial transmission studies were performed as described in section 3. At the end of this study the approximate $SMLD_{50}$ of virus contained per tick was also estimated in the manner previously outlined.

Miscellaneous

A study on transmission to chicks by contamination and contact was conducted with virus stock 2560-61. Normal chicks, half-day-old, were force-fed infected feces of viremic chicks; then they were placed in the same brooder with the known infected chicks and watched closely for ten days for signs of infection.

G. VIRUS PERSISTENCE IN THE HERON *ARGAS*

Several workers have cautioned that immune blood in naturally engorged infected ticks may neutralize the virus they contain when ground into suspensions for testing (Blaskovic and Rehacek, 1962). As a result, an erroneously low infection rate may be obtained for field-collected ticks which have been associated with the usually immune natural host.

Two experiments were performed to test for the possible effect of antibodies in the tick's blood meal upon the virus in infected ticks. The first was conducted with ticks from the July, August and December collections. Part of these were tested for virus as soon as received from Egypt, within 10-20 days after collection; the remainder were tested after an incubation period of six months at 27°C and 75% RH. By this time most of the natural blood meal was digested and antibodies unquestionably denatured. The natural infection rate of these ticks before and after the incubation was compared.

The second experiment acted as a control for the first one. Seven known virus-transmitting ticks were fed upon an immune cattle egret whose serum had been shown to neutralize $10^{6.0}$ PFU/ml of virus in a tissue culture neutralization test. A comparable number of similar ticks were retained unfed. After ten days of incubation the ticks of each group were tested individually for virus (1 tick/ml of diluent) and results compared. Part of the tick suspensions were titrated in suckling mice, to permit better estimation of effects of the immune

blood meal upon the virus. The ten-day period was selected since that was the shortest time that had elapsed between collecting ticks in the field in Egypt and processing them at the CDC.

III. OBSERVATIONS AND RESULTS

A. ARGAS (PERSICARGAS) ARBOREUS, NEW SUBGENUS, NEW SPECIES

1. Introduction:

Ten years ago, Dr. H. S. Hurlbut, working on tick viruses in the NAMRU-3* entomological laboratories, noticed that F_1 larvae of "*Argas persicus*" from arboreal rookeries of the Cattle Egret, *Bubulcus ibis ibis* (L., 1758), near Cairo were negatively geotropic while those from fowl houses were positively geotropic. Dr. Hurlbut observed also that F_1 larvae from these sources showed a predilection for the host species with which their parents had been associated (Hoogstraal, 1956).

During the course of this study it was found that the heron parasites do form a distinctive species that can be differentiated from *Argas persicus* (Oken, 1818) by morphological characters of adults, nymphs, and larvae. A report that was intended only to provide a name and description for the new species and to establish priority, according to the Rules of the Zoological Nomenclature was published with the collaboration of Dr. Harry Hoogstraal (NAMRU-3*) and Mr. Glen Kohls (RML)** (Kaiser *et al.*, 1964).

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2. Abstract of Publication:

Argas (Persicargas) arboreus new subgenus, new species, is very numerous in rookeries of the Buff-backed Heron (Bubulcus ibis ibis) near Cairo, Egypt, and differs from the cosmopolitan Argas (Persicargas) persicus (the type species of the new subgenus) in distinctive morphological features of adult, nymphs, and larvae as well as in biological characteristics and patterns of virus and Wolbachia infection. Adults, nymphs, and engorged and unengorged larvae of this species are described and illustrated. Both these species are compared with Argas (P.) beklemischevi Pospelove-Shtrom, Vassilieva, and Semaschko, 1963, described from five engorged larvae from a vulture in Turkmenia, U.S.S.R., and with A. (?P.) bureschi Drenski, 1957, known only by three adults from the nest of a ground squirrel in Bulgaria.

Persicargas new subgenus (i.e. the "persicus group") is characterized by the presence of lateral integumental cells and of postpalpal bristles in adult and nymphal stages. In the subgenus Argas (i.e. the "reflexus group"), the lateral integument is either striated only or mixed striated-celled, and postpalpal bristles are absent.

3. Holotype:

Female, from bark of an acacia tree supporting nests in rookery of Buff-backed Heron, Bubulcus ibis ibis (Linnaeus, 1758), in Nile Barrage Park, 18 miles north of Cairo, Qalyubiya Province, Egypt, U.A.R., 4 June 1963, Harry Hoogstraal (HH 20,200). Deposited in collections of

Rocky Mountain Laboratory (RML 38913).

4. Allotype:

Male; data and depository as for holotype.

5. Paratypes:

200 ♂♂, 200 ♀♀, 200 nymphs, 200 larvae, data same as for holotype. Paratypes are deposited in collections of the Rocky Mountain Laboratory, British Museum (Natural History), Entomological Society of Egypt, Cairo University (Faculty of Science, Department of Entomology), Chicago Natural History Museum, Institute of Malaria and Medical Parasitology (Moscow), University of Maryland (Department of Zoology), Musee Royal de l'Afrique Central (Tervuren), Division of Veterinary Services (Onderstepoort), East African Veterinary Research Organization, Institute of Parasitology and Malarialogy (Teheran), Old Dominion College (Department of Zoology), California Academy of Sciences, Harry Hoogstraal, and others.

B. VIRUS ISOLATION

Egyptian field collections of *A. arboreus* and *A. persicus* made from March through December, 1963, and January and February, 1964, were processed for virus isolation. The number of pools of *A. arboreus* tested of each sex or stage was ten (of five ticks each) and that for the *A. persicus* was five (of ten ticks each). Thus there was a total of 200 *A. arboreus* ticks tested in 40 pools* and 200 *A. persicus* ticks

*300 ticks in 60 pools in December 1963.

tested in 20 pools from each monthly collection. One hundred and fifteen virus isolations were recovered from 500 pools (2500 ticks) of *A. arboreus* representing monthly field collections for a year. In most instances virus isolations were confirmed by passaging in suckling mice. No virus was recovered from 2400 *A. persicus* tested in 240 pools.

C. VIRUS IDENTIFICATION

Sucrose-acetone and 10% seed virus antigens, live virus and BPL-killed virus vaccines, and immune and hyperimmune chicken and mouse sera were prepared for each prototype virus for the purpose of identification. In the CF screen tests sucrose-acetone antigens of each prototype used with broad group A, B and Bunyamwera antisera did not fix complement. The same antigens were also used with their homologous antisera and with hyperimmune mouse sera prepared against the ungrouped viruses of Quaranfil, Chenuda, and Nyamanini. In these tests the stock 2558-59 virus prototype reacted strongly with specific Nyamanini antiserum and with its own homologous system. Virus stock 2560-61 strongly fixed complement with specific Quaranfil antiserum and its own homologous system. In both cases no cross-reaction was observed with Chenuda antisera. Thus, virus stocks 2558-59 and 2560-61 were proved identical to Nyamanini and Quaranfil viruses, respectively, and antigenically unrelated to each other or to Chenuda virus.

Crude CF antigens were prepared from each of the 115 mouse brain isolates and employed for identification by the micro CF technique.

Out of these, 73 fixed complement with each of Stock 2558-59 and Nyamanini hyperimmune mouse sera. The remaining 42 reacted with Stock 2560-61 and Quaranfil hyperimmune mouse sera. This result not only identified the isolates but also confirmed the identity of prototype virus stocks as Nyamanini and Quaranfil.

The 42 tick pool suspensions judged on the basis of CF tests to be infected with Quaranfil virus (= prototype 2560-61) were tested by the adsorption method for possible concurrent presence of Nyamanini virus (= prototype 2558-59). The tests were performed in duck-embryo tissue cultures and in suckling mice. Three tick suspensions were found Nyamanini-positive after adsorption with immune chicken serum 2560-61. All three were from the November, 1963, collection and carried the designation numbers ♂ (26-30), ♀ (36-30) and N_2(11-15) (Table II). Results were confirmed by passaging plaques and mouse brains in suckling mice. Adsorption test controls showed each virus to be plaque-producing and to be neutralized by its own immune serum. The antibody level in each antiserum utilized in the test was measured in Log_{10} PFU of protection against the virus per ml of antiserum. Immune chicken serum 2560-61 gave 4.8 logs of protection against Quaranfil virus, and 6.3 logs against its homologous virus. Immune chicken serum 2558-59 gave 3.5 logs of protection against Nyamanini virus, and 5.3 logs against its homologous virus. No inhibition of viruses was observed with the heterologous antisera. Results obtained with mixtures of equal parts of prototype stock viruses and 1:1 mixtures of their immune sera were consistent with these observations.

Quaranfil, stock 2560-61, Nyamanini and stock 2558-59 viruses titered 4.8, 6.3, 6.1 and 5.3 Log_{10} PFU/ml, respectively. The end result of the adsorption test was the addition of 3 more isolates of Nyamanini virus, bringing their number to 76, and the total number of isolates, both viruses combined, to 118.

To confirm the virus identifications made by CF tests, 8 isolates were selected for checking also by cross-neutralization tests conducted in duck-embryo tissue cultures. The results agreed with those obtained by CF (Table III). Each of the antisera employed in the test gave greater than $10^{2.0}$ PFU protection against its specific virus (or isolate) per ml of antiserum; a protection of 2 logs or greater is considered significant. These results were obtained by substracting the number of Log_{10} PFU of virus obtained with its homologous serum from that obtained with normal serum. In these tests no cross-reactions between heterologous systems were detected.

In Table IV the number of pools infected is indicated for each monthly field collection of _A. arboreus_ tested. From these data, the pool and tick infection rates were obtained.

Natural tick infection rates per cent (Table IV) were calculated according to formula for estimation of infection rates in arthropods (Chiang and Reeves, 1962). For example, if 7 pools of 5 ticks each out of 40 such pools are positive, 33 out of 40 are negative. Then $(1-X)^5 = 33/40$, the proportion where every pool is negative.

$$1 - X = (33/40)^{1/5} \qquad \text{Log } 33 = 11.5185 - 10$$

$$\text{Log } 40 = 1.6021$$

$$1 - X = \text{anti-log } \frac{9.9164-10}{5} \qquad 9.9164 - 10$$

$$\text{or anti-log } 1.9833-2$$

$$\text{or anti-log } 9.9833-10$$

$$= 0.9622$$

$X = 1-0.9622 = 0.0378$ or 3.78% natural tick infection rate.

The natural infection indexes of *A. arboreus* were determined for Nyamanini virus, Quaranfil virus, and both viruses combined (figs. 1 and 2). The natural infection index is defined as the per cent of infected arthropod vectors in a natural population, based on observed infection rates in field samples at different times of the year (Jenkins, 1962). For Nyamanini virus, it was highest in June and lowest in September and December of 1963. It had two other shorter peaks, in October, 1963, and in February, 1964. Quaranfil virus had the highest peak in March, 1963, with an almost abrupt drop to reach its lowest level in June and July. This level was maintained with a slight rise during late summer and early fall. A moderately high level in November graduately lowered through December, 1963, and January and February, 1964.

Virus isolations from *A. arboreus* according to sex, nymphal stage and amount of residual blood in stomach and diverticula at the time of testing are listed in Table V. No significant difference was apparent between the percentage of virus positive pools of ticks con-

taining "++++" x "+++" of residual blood at time of testing and that of pools of ticks containing "+" x "-" amounts of residual blood.

D. VIRUS CHARACTERIZATION

1. Susceptibility of animals to Nyamanini virus (prototype 2558-59)

Wet virus and reconstituted seed virus titered $10^{6.8}$ and $10^{4.2}$ $SMLD_{50}$/ml respectively. Suckling mice consistently died 7 to 8 days after inoculation with 0.02 ml i.c. of a 10^{-2} dilution. Neither 3-week old mice nor 0.5 day-chicks were susceptible by the i.c. and i.p. routes after inoculation with 0.03 ml of a 10^{-2} dilution. Chickens, 5 weeks of age, also proved insusceptible to the virus after inoculation with the same amount and concentration of virus by the s.c. route.

2. Susceptibility of animals to Quaranfil virus (prototype 2560-61).

Wet virus and rehydrated seed virus titered $10^{8.0}$ and $10^{7.2}$ $SMLD_{50}$/ml i.c., respectively. Suckling mice consistently died 3 to 4 days after inoculation with 0.02 ml i.c. of a 10^{-2} dilution of virus. The seed virus also titered $10^{7.0}$ LD_{50}/ml in 3 week-old mice inoculated via the i.c. route. By the i.p. route these mice were resistant to infection; only two out of six inoculated with 0.03 ml of a 10^{-2} dilution died on the 9th and 10th days following inoculation. Chicks, 0.5 day old, inoculated i.c. and i.p. with the same amount and concentration of seed virus died, following a few hours of weakness and prostration, after an incubation period of 4 to 5 days. Five-week-old chickens and adult herons inoculated s.c. with the same dosage were not

affected. Wet virus titered $10^{7.0}$ LD_{50}/ml in 0.5-day-chicks by the s.c. route, one log less than in SM, i.c. No more than 10-100 i.c. $SMLD_{50}$ units of virus are required to infect a chick by s.c. route.

3. Viremia studies in baby chicks with Nyamanini and Quaranfil viruses

Nyamanini virus did not produce a detectable viremia in six chicks inoculated s.c. with approximately 3,000 i.c. $SMLD_{50}$ units. All chicks lived until they were exanguinated for immune blood two months following the inoculation.

Eight chicks were inoculated with 3,000 i.c. $SMLD_{50}$ units of Quaranfil virus by the s.c. route. Six were bled daily; the other two were reserved for bleeding in the event of deaths in the first six. One of the 6 developed a viremia on the first through the fourth days following inoculation. Daily viremia levels expressed in Log_{10} i.c. $SMLD_{50}$/ml of chick serum were 2.3, 3.7, 3.7 and 5.0, respectively. This chick died on day 5. On the fourth day four other chicks developed viremia at the levels of 2.3, 2.5, 4.0 and 5.0 logs; the latter three died on the following day. The remaining one circulated 3.4 logs of virus on day 5 and died of infection on day 6. On the fifth day following inoculation two chicks had viremias of 4.2 and 3.2 logs. The first died on day 6; the second maintained the same level of viremia through day 6 and died on day 7.

	Day 1	D2	D3	D4	D5	D6	D7
Ch 1	0	0	0	X			
Ch 2	0	0	0	4.0	X		
Ch 3	2.3	3.7	3.7	5.0	X		
Ch 4	0	0	0	2.5	X		
Ch 5	0	0	0	2.3	3.4	X	
Ch 6	0	0	0	0	4.2	X	
Ch 7	-	-	-	5.0	X		
Ch 8	-	-	-	-	3.2	3.2	X

VIREMIA LEVELS EXPRESSED IN LOG_{10} I.C.

$SMLD_{50}$/ML of CHICK SERUM

X = Dead - = not bled 0 = no virus detected in 1:10 dilution of plasma

The study indicates that the peak of viremia in baby chicks is reached on the fourth and fifth days.

4. Viremia study in adult herons with Quaranfil virus.

A one-year-old cattle egret, *Bubulcus i. ibis*, developed a low grade viremia on the third day following s.c. inoculation with 3,000 $SMLD_{50}$ i.c. units of virus. No virus was detected on the later days and the bird survived the infection. No viremia was detected in another cattle egret and two snowy egrets of similar ages inoculated with the same amount of virus.

5. Behavior of Nyamanini and Quaranfil viruses in tissue culture.

Duck-embryo monolayer tissue culture cells inoculated with

Quaranfil virus started to develop cytopathogenic effect (CPE) beginning the 3rd day after inoculation. The virus induced focal areas of necrosis (microplaques) which were not effectively readable till the 5th day; the greatest numbers of plaques were not reached until the 7th or 8th day (fig. 19). From these plaques, infections spread progressively to neighboring cells, causing destruction of the whole cell sheet on or about the 11th day. The Quaranfil virus plaque was typically irregular, 1-2 mm in diameter, with complete degeneration of the cells within its area.

When duck embryo tissue cultures were inoculated with Nyamanini virus, individual cell necrosis started to appear on the fifth day and gradually disseminated through the monolayer within the next 36 hours. The greatest count of plaques was reached on the 10th day following inoculation. Cultures exhibited complete destruction on the 11th and 12th days. Nyamanini virus plaques showed irregular necroses within their areas, with less on the periphery where it was more diffused, giving an opaque appearance. Plaques were more regular in outline than those of Quaranfil virus but were similar in size, also measuring 1-2 mm in diameter.

6. Hemagglutinating activity of Nyamanini and Quaranfil viruses.

The two viruses were tested for ability to hemagglutinate goose-red cells at pH readings of 5.7, 6.0, 6.2, 6.4, 6.6 and 6.8. The results were negative.

E. LIFE CYCLE OF *ARGAS ARBOREUS* (TABLE I)

The life cycle under insectary conditions of 27°C and 75% relative humidity required about three months. When the ticks were reared under ordinary room conditions, the incubation periods required for oviposition and development varied inversely with the temperature. With the females from the December, 1962, collection, oviposition commenced from 10 to 75 days after they were fed on chicks and paired with males; the median period was 50 days and the mean 47.2 days. In all subsequent feedings, made on pigeons, the oviposition started 4 to 11 days after engorgement with a median of 5 days and a mean of 6.0 days. Usually, a female required 3 to 4 days to complete the deposition of a relatively large batch of eggs, from the first egg to the last.

The number of eggs laid after the first feeding generally ranged from 15 to 80; in one case, however, 200 were produced. The number of eggs laid after subsequent feedings increased progressively: 60 to 100 for second feeding; 50 to 180 for the third feeding; and up to 260, the greatest number obtained from a single individual, after the fourth feeding. Other factors influencing the number of eggs were found to be the size of the tick, the extent of engorgement and the presence of the male. Unmated females remained several weeks after engorging without depositing eggs, but commenced to do so a few days after being placed with males. In some instances mated ticks did not oviposit until they had fed a second time, and, rarely, for a third

time. During these long preoviposition periods these females flattened considerably, the blood evidently being used to sustain life rather than to produce eggs.

Although almost invariably a blood meal was required by the females for oviposition, in one exceptional case a female which had molted from the third nymphal stage laid two viable eggs. Apparently, sufficient food reserve was carried over from the nymphal stage for production of these eggs. This was apparently not an example of parthenogenesis since the female had been associated with males.

After oviposition, the females "brooded" over the eggs, a phenomenon of unknown function common among argasids. The newly laid eggs were slightly ovoid and a glistening golden yellow in color. They gradually changed color to amber-brown and became more turbid in the following few days. After the sixth day, the developing embryo could be seen through the waxed cuticle as an undifferentiated mass of white material. The larva was distinguishable 2 to 3 days before hatching.

The period required for the incubation of eggs was 11 to 18 days at 27°C (median, 16 days; mean, 15.7 days). Larvae emerged by alternate contractions of the anterior and posterior ends of the body, an action which ruptured the shell and exposed the larval dorsal surface.

The best feeding results were obtained when larvae were placed on a host 8 to 11 days after hatching. Earlier attempts were less successful, presumably because the mouth parts had not hardened.

Beyond 11 days, progressively poorer feedings resulted from weakness due to starvation.

Ordinarily larvae did not commence to drop from the host until the fifth day following attachment, although occasionally a few dropped a day sooner. All the larvae observed which engorged normally left the host by the eleventh day following attachment (median, 7 days; mean, 6 days).

A developing resistance in the host against the bite of the tick appeared to be a major factor acting against the engorgement of slow-feeding larvae. Most of such larvae, although satisfactorily attached, could not draw blood and usually died and withered _in situ_. Others became filled with a pink fluid rather than blood; these detached after a comparatively long time, but usually did not molt to the first nymphal stage.

When more than 25 larvae fed together on a single pigeon, it became paralyzed and died on the fourth or the fifth day following larval attachment. This paralysis was more evident with larvae of the optimum age group of 8 to 11 days. By the time of the pigeon's death about 20 of the larvae would have completed feeding and dropped off. The remainder would be in various stages of repletion or already exhibiting effects of resistant-host feeding.

The larval stage is the only stage in which the species remains upon the host for any length of time. Engorged larvae dropped off only at night and assumed a flattened shape a few hours after detaching.

Under insectary conditions, unfed larvae of _A. arboreus_ lived for 23 to 38 days, but those over 20 days of age were so weakened that they never successfully completed engorgement. Larvae of another species of _Argas_ (_A. persicus_), on the other hand, survived unfed for over three months.

Molting to the first nymphal stage (N_1) commenced as early as the fourth day following larval repletion and as late as the fourteenth day (median, 7 days; mean, 7.4 days). The nymphs freed themselves from the exuvium through an anterior slit. Unlike larvae, they usually fed only 10 to 15 minutes; rarely they attached for 2 to 3 days but were probably not feeding during that time. Without feeding they lived about 4 months.

Molting to the second nymphal stage took place in about 12 days after feeding (median, 11 days; mean, 7 days). The second stage nymphs (N_2) were very rapid feeders; time for engorgement was only 2 to 15 minutes. If they did not have an opportunity to feed, they could survive over 8 months without apparent ill effects.

The time for molting of the second stage nymphs after feeding ranged from 10 to over 26 days. About three-fourths went directly from the N_2 to the adult stage. The remainder passed instead to a third nymphal stage (N_3) in which instance an additional period of approximately two weeks post-feeding by the N_3 stage was required to molt to the adult. The medians and means, respectively, in days, for each transtadial period were as follows: N_2 to adult, 13 and 13.8; N_2 to N_3, 14 and and 14.6; N_3 to adult, 15 and 15.5.

Of 113 individuals which went directly from the N_2 to adult stage, 77 (68%) were shown to be males and 36 (32%) to be females. A considerably different sex ratio was revealed in those undergoing an N_3 stage, however. Of 44 individuals observed, 37 (84%) became females and only 7 (16%) became males. Since an additional blood feeding was involved, these findings indicate a greater nutritional requirement to mature a female than a male. The unfed N_3 stage lived as long as adults.

The longevity of adults is very great. From a lot of approximately 400 ticks collected in March, 1963, which were kept in a rearing tube under insectary conditions, 3 were still alive in November, 1964, a period of 19 months. Similar numbers of ticks from earlier collections (December, 1962 and January, February, 1963) were all dead on that date. About a third of the ticks collected in June, 1963, were still living. Most of the field-collected ticks lived for more than a year. The longevity of adults that had not fed was less than those which had. Also, females which oviposited survived unfed for a shorter period than males and non-laying females.

The adults engorged repeatedly and females usually oviposited following each engorgement. Males would feed readily about every 50 days. Females would feed every 25 to 30 days, or oftener. However, in instances where oviposition did not take place, the female would refuse to refeed, even beyond 30 days. As shown in Table I, females were permitted to engorge as many as four times, with each engorgement followed by oviposition. The time required for engorgement ranged from

10 to 40 minutes, rarely as long as 90 minutes. Observations showed that in general males became replete faster than females.

Coxal fluid was usually emitted after feeding and detaching; in rare instances this occurred before detaching. In one case, a female discharged a few drops of dark blood from the anus after feeding.

Sometimes ticks were seen copulating immediately after feeding. Copulation took place whether males were fed or not and lasted for about 15 minutes.

Usually best results of feeding were obtained when ticks were fed at night or under a dark cover, but for the purpose of these observations, the host was tied down and the ticks fed under indirect light.

F. EXPERIMENTAL INFECTION OF ARGAS ARBOREUS AND ARGAS PERSICUS WITH QUARANFIL VIRUS AND THE VIRUS OF ST. LOUIS ENCEPHALITIS

Since the life cycle of *A. arboreus* took three months, a period of over six months was required to build up four colonies for experimental use after proving the stock to be "clean" (naturally virus free). Field-collected *A. persicus* ticks were considered normal (clean) without prior testing (see Section E).

1. Transmission study with Quaranfil virus in A. arboreus.

Argas arboreus ticks were fed upon infected chicks circulating Quaranfil virus in titers of $10^{3.3}$ to $10^{5.7}$ SM i.c. LD_{50}/ml of serum. After different extrinsic incubation periods (EIP), the ticks were tested for ability to transmit by bite, either singly or in pairs

(Tables VI and VIII). Twenty-one ticks out of 29 (72.4%) which were refed upon normal chicks after 23 to 36 days EIP induced immunity. The 30-day post-feeding chick sera neutralized $10^{4.9}$ to $10^{6.3}$ PFU of virus per ml of serum. One tick (3.4%) was fatally infective; its host chick died on the sixth day following the transmission feeding. After 43 to 95 days EIP, 9 ticks out of 25 (36%) were fatally infective; only one (4%) induced immunity (Table VIII).

Results obtained by periodical testing of coxal fluids for virus (Table IX) correlate in part with the above data. None of 17 coxal fluids of single ticks tested before 44 days of EIP was found virus positive. Virus was isolated from 8 out of 15 (53.3%) of coxal fluids examined on and after this period. Virus in the coxal fluid is taken as an indication of widespread infection in the tick, beyond the digestive tract.

Ninety-four per cent (33/35) of the ticks which were processed singly for virus isolations were found infected, a very high infection rate. Tests were performed during and at the end of the study after EIP ranging from 23 to 95 days. All tick suspensions tested killed 6/6 of suckling mice inoculated, indicating presence of considerably more than mere traces of virus in the ticks. Of representative ticks titrated, the smallest amount of virus found per tick was $10^{3.1}$ SM i.c. LD_{50}; the highest was $10^{5.7}$.

Experimentally infected nymphs that molted to subsequent nymphal stages or to adults maintained infection up to 88 days following the infective meal despite ecdysis. However, one female that had

originated from a third-stage nymph was negative for virus after 88 days of EIP.

Whole batches of larvae from the first, second, third and fourth ovipositions were all found negative for virus (Table VII). These larvae were the offspring of either experimentally infected females paired with infected or normal males, or normal females paired with infected males. Therefore, no evidence of transovarial or transspermatophoral transmission was indicated.

2. Transmission study with Quaranfil virus in A. persicus

Normal fowl ticks were permitted to engorge upon experimentally infected chicks circulating Quaranfil virus in titers of $10^{4.3}$ to $10^{5.7}$ SM i.c. LD_{50} per ml of serum. Subsequently, after EIP ranging from 33 to 86 days, they were tested for the ability to transmit by bite (Table X). Refeedings were made on single normal chicks, either in mass (Tr-stocks 22, 42, 43 and 44) or in pairs. The normal chicks fed upon by Tr-Stock 22 after EIP of 47 days and 86 days died of infection 5 and 11 days later, respectively. This stock consisted of 23 ♂♂ and 5 ♀♀. One normal chick fed upon by Tr-stock 42 (10 ♂♂ and 1 ♀) after EIP of 33 days, was injured in handling so was bled on the third day. In this blood sample a low titer of virus was detected, sufficient only to kill one out of six suckling mice inoculated. No transmission was effected by bites of experimentally "infected" ticks refed in pairs; also, no apparent induction of immunity was demonstrated in the chicks they refed upon.

At different intervals extending from 12 to 106 days EIP, coxal fluids of single ticks were tested for virus (Table XI). One coxal fluid out of 25 (4%) was found to contain an extremely low concentration of virus. The fluid had been extracted from a ♂ tick from Tr-stock 43, 92 days following the initial infected meal.

Samples of ticks used in the transmission study were screened periodically for the presence of the virus, (Table X). From the results the infection rates for ♂♂, ♀♀ and NN (after ecdysis) were determined to be 34.4% (11/32), 55.6% (5/9), and 85.7% (6/7), respectively. The overall rate of infection, regardless of sex or stage, was 45.8%. The amount of virus contained per tick at different EIP ranged from $10^{2.5}$ to $10^{4.3}$ SM i.c. LD_{50}. Batches of F_1 larvae obtained from the first and second ovipositions of experimentally "infected" female were all negative when ground and tested for virus (Table XII).

3. St. Louis encephalitic virus transmission attempts with *A. arboreus*

Six males, 11 females and 9 nymphs were fed on infected baby chicks circulating St. Louis encephalitis virus in titers ranging from $10^{5.2}$ to $10^{5.5}$ SM i.c. LD_{50}/ml of serum. Ticks of this "infected" group which refed upon normal chicks 24 days and 49 days following the infected meal failed to transmit the virus (Table XIII). After periods of 24 and 52 days of EIP, the rate of infection of the ticks was found to be 20% (1/5) and 19% (4/17) respectively. The overall rate of infection was 19.2% (5/26), taking into consideration that the six males were found negative after 52 days EIP and only one nymph out of

9 that molted harbored the virus on the 24th day. The maximum SM i.c. LD_{50} of virus contained per tick was $10^{5.5}$, as determined in a female titrated after an EIP of 52 days.

Twenty batches of F_1 larvae from the first and second ovipositions of experimentally "infected" parents were found negative for virus (Table XIV).

4. Study on the effect of Quaranfil and SLE virus infections on the life cycle of *A. arboreus*.

No significant differences were observed between the parasitic periods of normal, non-infected ticks and those of the experimentally infected ticks.

5. Study on transmission of Quaranfil virus to chicks orally by contamination.

Quaranfil virus was present in titers of $10^{4.0}$ to $10^{4.3}$ SM i.c. LD_{50}/ml in the stools of viremic chicks tested on the sixth day after S.C. inoculation with the virus. Normal baby chicks which were force-fed portions of these infected stools did not exhibit overt symptoms up to the tenth day following this attempt at infection by contamination.

G. STUDIES ON VIRUS PERSISTENCE IN *A. ARBOREUS*

Natural Quaranfil virus infection rates* of the July, August and

*Calculated

December, 1963, field-collected ticks, determined after 6 month's laboratory incubation, were 1.0%, 1.4% and 2.5%, respectively. In comparison, infection rates of 0.5%, 1.0%, and 2.5% were obtained from comparable pools of ticks tested directly after receipt of shipments, only 10 to 20 days after collection (fig. I). Natural infection rates with Nyamanini virus after six month's laboratory incubation were 1.0% for July, 2.8% for August and 2.5% for December, as compared to 4.4%, 2.6% and 1.7% obtained, respectively, when field material was processed directly. The overall infection rates with one or the other of the two viruses were, after the 6 month's incubation, 2.1% for July, 4.4% for August and 5.2% for December; the rates in those not so incubated were 5.0%, 3.8% and 4.4%, respectively.

The after-incubation results were obtained by processing 15 pools of five ticks each from each monthly collection. Since the longevity of the first stage nymphs does not exceed 4 months, they did not survive the six-month incubation period and subsequently were not included in this test.

These results indicate that the probable presence of antibody in the residual blood in field-collected ticks apparently had little or no diminishing effect upon detection of viruses in ticks. These field results are further confirmed by the finding that immune blood meals did not have a noticeable adverse effect on detection of virus in experimentally infected ticks tested 10 days following their virus meal. Individual tick suspensions had sufficient active virus to kill 6/6 suckling mice within 5 to 6 days after inoculation. The virus

titered 4.5 $SMLD_{50}$ i.c. in one female and 3.5 logs in one male quantitatively checked (Table VI).

IV. DISCUSSION

In the symposium "The Species Problem", published in 1957 by the American Association for the Advancement of Science, Ernst Mayr says:

> The species is a biological phenomenon that cannot be ignored. Whatever else the species might be, there is no question that it is one of the primary levels of integration in many branches of biology, as in systematics (including that of microorganisms), genetics, and ecology, but also in physiology and in the study of behavior. Every living organism is a member of a species, and the attributes of these organisms can often best be interpreted in terms of this relationship. This is particularly true in comparative studies.

He concludes that,

> species are important because they represent an important level of integration in living nature. This recognition is fundamental to pure biology, no less than to all subdivisions of applied biology. Whether he realizes it or not, every biologist - even he who works on the molecular level - works with species or parts of species and his findings may be influenced decisively by the choice of a particular species. The communication of his results will depend on the correct identification of the species involved, and thus on its taxonomy.

Little real effort has been made to understand fully some of the pathopherous species, or to define precisely their range and limits of variation. Evaluation and practical application of results of some of incidental and monumental taxonomic, biologic and epidemiologic studies, referring to erroneous or unrecognizable species appellations, have a limited value owing to the uncertainty of the species with which they deal. Applied researchers overlooking the necessity of

being able to identify, describe, and differentiate their experimental species, have wasted both considerable time and effort.

On the other hand,

> there are many taxonomic problems still to be solved that transcend strict morphological criteria and impede confident delineation of pertinent vector relationships (C. B. Philip, 1963).

Thus, taxonomists, dealing with preserved specimens alone, are hopelessly unable to define these species for the first time, as may be the case with the *Argas persicus* complex. Biologists and epidemiologists lacking adequate systematic support are under great handicap. On the other hand, by teamwork and close correlation of systematic and biologic studies in the laboratory and in the field, taxonomic problems can often readily be solved. The properties of parasites discovered by the immunologists, physiologists, and biochemists must be incorporated in the systematic pattern (Hoogstraal and Kaiser, 1958; Wharton, 1962); epidemiologic evaluation then becomes more certain.

Since the discovery and description of *A. arboreus*, and the erection of the subgenus *Persicargas* (Kaiser *et al*), it has been realized that *Argas persicus* was used for many years as a "catch-all" name by many workers around the world. Now, that the needed criteria have been defined, closely related species that differ equally as much in their relative importance as vectors of certain pathogens are being discovered. Several viruses have been isolated from a species, now being described, from Lahore by the Pakistan Medical Research Center (Hoogstraal, personal communication). Also, the writer believes that one or more undescribed or previously synonymized species occur in the

Americas in lieu of A. persicus.

The zoogeographical range of the newly described A. arboreus has now been extended to include Tanganiyka and South Africa. Although the Old World cattle egret has established itself in parts of Florida, and on the Mexican Gulf and Atlantic coasts since 1940 (Palmer, 1962), the Argas arboreus tick usually linked with its rookeries was not recovered. The Tampa, Sarasota, Miakka River, and St. Petersburg areas of Florida and the Okefenokee Swamp of Georgia were surveyed for the presence of this species in cooperation with Dr. Telford Work, Chief of the Virus and Rickettsia Section, CDC, and Dr. W. D. Burbanck, Professor at the Department of Biology, Emory University, with negative results.

Studies on the rates of infection in the monthly collections of A. arboreus and A. persicus from Egypt have further confirmed the differences in field infections manifested by the pilot investigations. These differences are now attributed to host-parasite relationships of two distinctive, although related, species rather than the genetic susceptibility of two populations of the same species. This does not imply that the host-parasite system, mentioned here in its broad sense, does not also include the factor of susceptibility of host cells of a particular species to the invading intracellular parasite. Contrary to the case with A. persicus in this study, finding an arthropod to contain virus under natural conditions cannot be interpreted as complete evidence that the species is a vector. Practically any hematophagous arthropod might upon occasion receive a virus into the

digestive tract, but the capacity of the cells lining the gut to allow multiplication of the virus and the infection of the salivary glands via the haemocoele is part of the properties of certain species. In the opinion of Rehacek (1965) the ability of a poikilothermic arthropod to become a biological vector is first determined genetically in the course of the phylogenesis of the species.

In opposition to this view, Smith (1964) considers that ecological factors have a first hand in determining the species of an arthropod host, "...although a limited degree of specificity between viruses and arthropods exists." In early studies of vector-virus relationships it was thought that a high degree of generic and species specificity existed. Recent observations do not necessarily support this belief. Some viruses were experimentally transmitted by more than one species of vector, often from different genera and sometimes from different families or orders. For example, Chumakov _et_ _al_. (1945) demonstrated the infection of _Ixodes_ _ricinus_, _Dermacentor_ _pictus_, _D_. _nuttali_, _Hyalomma_ _dromedarii_, _H_. _asiaticum_ and _H_. _turkmeniense_ with the virus of Russian spring-summer encephalitis (RSSE). Transovarial transmission was observed in the three tick genera. However, the natural vector of RSSE is known to be _Ixodes_ _persulcatus_. Mosquito-borne viruses such as those of Western equine and St. Louis encephalitis may be transmitted experimentally by _Dermacentor_ _andersoni_ and _D_. _variabilis_ ticks (Syverton and Berry, 1941; Blattner and Heys, 1944). Transmission of West Nile virus (mosquito-borne) by _Ornithodoros_ _coniceps_ ticks was reported by Vermil

et al. (1958, 1960). Schmidt and Said (1964) recovered this virus from *Argas reflexus hermanni* collected in midwinter. They speculated that this tick serves as an overwintering vector (reservoir) and thus alternates with the seasonal culicine vectors, *Culex antennatus* and *C. univittatus*, in the natural maintenance of the virus between seasons.

On the other hand, with some viruses there is considerable vector specificity. For example, not all mosquito-borne viruses appear to be equally infectious for ticks. Whitman and Aitken (1960) found that Semliki Forest, Bunyamwera and Oriboca viruses did not persist in *Ornithodoros moubata* when infected by feeding. Although the ticks were infectible with West Nile virus, transmission was accomplished only by mass feeding, and the authors were inclined to believe this transmission to be through skin contamination with virus in the coxal fluid rather than by bite. The virus of Colorado tick fever appeared unable to infect mosquito tissues (Eklund, 1954).

Apparently a high degree of specificity exists between Quaranfil virus and the vector species, *A. arboreus*. The high transmission and infection rates obtained in the experimental studies with this tick demonstrate an excellent vector potential rating (Chamberlain *et al*., 1954). The poor-to-moderate infection rate obtained with *A. persicus* is not in itself a proof of vector efficiency since transmission of the virus was only effected by mass feeding of ticks on single hosts. Either the quantity of virus inoculated by pairs of *A. persicus* ticks during feeding was not sufficient to cause the infection, thus

requiring several ticks to inject virus of a sufficient concentration to guarantee a transmission, or only a relatively low proportion of the ticks had infected salivary glands.

Contrary to the finding by Whitman and Aitken (1960) with O. moubata, Quaranfil virus was not demonstrable in the coxal fluids of A. persicus, except in one case where it was detected in a very low concentration. Thus, it is tempting to speculate that the low concentration of virus is transmitted mechanically through regurgitation of residual infected blood in the tick, rather than biologically via the salivary glands. However, it is equally possible that an occasional individual of A. persicus becomes biologically infective and transmits by bite.

Philip (1930) discussed and ruled out the possibility of mechanical transfer of viruses by regurgitation during intermittent feedings of mosquitoes. His thesis may in part be supported by a recent study on the sequential events that take place in the mosquito alimentary canal during digestion of a blood meal (Howard, 1962). The fact that in the female mosquito the meal becomes a solid clot in less than an hour after feeding and that the digestion is thoroughly completed at 72-120 hours may account for fast destruction of free virus. Also, the possibility exists that the structure of the mouth parts and oesophagal diverticulae of mosquitoes does lend itself to the transfer of virus by regurgitation during the act of feeding (Philip, 1930). There is strong indication, however, that mechanical transmission by contaminated mouth parts of mosquitoes may be accomplished for at

least 20 hours (Chamberlain and Sudia, 1961).

Recent histological and histochemical studies on digestion of blood in A. persicus have shown that ingestion of blood is accompanied by the disintegration of epithelial cells lining the gut. After hemolysis caused by enzymatic secretions of the newly developing epithelium has occured, digestion takes place in two phases. The first is rapid and lasts approximately 1 to 2 weeks and is followed by a second phase which is slow and remains at a constant rate for several months until feeding or death occurs (Tatchell, 1964). For the purpose of this study and because of the epidemiological implications of the problem of regurgitation by ticks, the writer consulted Dr. J. D. Gregson, Chief of the Veterinary and Medical Insect Section, Canadian Department of Agriculture. Dr. Gregson wrote:

> Upon watching the feeding of the tick, Dermacentor andersoni, on transilluminated membranes there occasionally appeared from the hypostome puffs of blood that seemed to come from the tick, although there was the possibility that the phenomenon was due to free blood being picked up in the outbursts of salivary secretion. Further observations on ticks that were being fed artifically left no doubt that the blood was regurgitated. It is of a brighter red color, and quite voluminous at times, too much, I'd say, to come only from the pharynx. Whether the same might be true with Argas ticks I cannot say, but there does seem a possibility. (Gregson personal correspondence, December, 1964).

Regurgitation of only partially digested, still infective blood from a previous meal by A. arboreus might well explain an unexpected finding in the present study. Approximately 72% of experimentally infected A. arboreus ticks which were refed in pairs upon normal chicks before 44 days of extrinsic incubation induced immunity. None

of the coxal fluids tested during this period was found virus positive indicating that generalized infection had not yet occurred in these ticks. It seems possible that residual virus could have been transmitted by regurgitation, and since it would be low in concentration because of the partial digestion, would tend to produce a subclinical immunizing infection rather than a fulminating one terminating in death. Perhaps, had these ticks been refed in mass upon single chicks they might together have been capable of transmitting fatal infections, as may have been the case with the mass-fed A. persicus. On the other hand, inactivated virus together with a low concentration of live virus in regurgitated blood might also have played a part in the immunizing process.

From the results presented in this study it is known that between 10 to 100 (approximately 30) i.c. $SMLD_{50}$ units of Quaranfil virus are required to cause infection and death of a baby chick. Further studies are needed to determine quantitatively the least amount of virus required to induce immunity. The results of such studies may also indicate whether death rather than immunization is probable through the cumulative effect of mass-feeding.

In the study with SLE virus, the fact that A. arboreus did not transmit during the test period of 52 days extrinsic incubation does not rule out the possibility that it may act as a reservoir to carry the virus over long periods. A certain proportion (19.2%) retained virus for at least 24-52 days. Had the ticks been held for a longer period, a few might have become able to transmit by bite. The

reservoir function is used in this study in the sense considered by Philip and Burgdorfer (1961),

> ...not a static but a dynamic and flexible adjustment in the relative importance of arthropod and vertebrate hosts in natural maintenance of a given microbial parasite.

Since epidemiologic and laboratory studies indicate that birds, wild and domestic, are natural hosts of the SLE virus (Sudia, 1959), a common bird ectoparasite such as an _Argas_ species might indeed serve as an effective reservoir. Although the findings reported here are not definitive, they suggest lines of further research. Experimental studies evaluating mosquitoes (Sudia and Chamberlain, 1964) and chicken mites (Smith _et al._, 1946 and Reeves _et al._, 1955) as reservoirs of SLE virus have been made, with the conclusion that these arthropods play no significant reservoir role. However, to date no reports have been published concerning _Argas_ ticks in this regard.

Virus isolations from field-collected _A. arboreus_ indicate that it qualifies as both reservoir and vector for Quaranfil and Nyamanini viruses. The finding that it is infected with both viruses the year round is a sufficient demonstration of this ability.

Three isolations out of 115 were identified as mixed infection with Quaranfil and Nyamanini viruses. The possibility of double infection with both viruses in single ticks seems unlikely, since the ticks were tested in pools of 5, and only approximately 2.5% of the infected pools contained both viruses. When a mixed-infection pool was inoculated into suckling mice, the faster virus suppressed the slower. Only when the faster virus (Quaranfil) was suppressed by

specific antibody, was the slower virus (Nymanini) allowed to progress and thus reveal these few mixed infections.

Figures I and II show the results obtained in the seasonal study of infection rates of _A. arboreus_ with Quaranfil and Nymanini viruses. Obviously, several interrelated factors are responsible for the picture obtained. The most obvious ones concern the tick, _A. arboreus_, the bird, _Bubulcus i. ibis_, and the climate.

The tick-related factors include its life cycle, longevity, rate of multiplication, biting habits and its potentiality to act as a vector and reservoir of the viruses. All of these factors have either already been discussed or can be inferred from the experimental data presented.

Transmission of the virus through birds depends upon the availability of a sufficient population of non-immune hosts (Smith, 1964). In an enzootic area, such as the Barrage, most of the adult cattle egrets probably have been infected at a younger age and consequently are incapable of producing viremia. And, since some of the non-immune nestlings are probably eliminated from the population by fatal infection, continued transmission of the virus must depend largely on the high reproductive capacity of these birds.

Field reports state that the egg clutch of cattle egrets ranges between 4 to 6 eggs and sometimes more. The birds arrive on the bleeding site in early March and start selecting positions for their nests. In April females brood their eggs, with the males standing beside them or bringing food. Tops of trees appear "white capped"

owing to numerous birds at this height. Young birds in various stages of development, some attempting to fly, are seen during May and June. They are infested by A. arboreus larvae all over the body as well as under the wings. During July and August many females are seen sitting in the nests again. In October no birds are seen in the nests but hundreds of flightless young are found on the ground under the trees. All birds leave the breeding site by the end of this month.

It has been long recognized that climatic factors exert a major influence upon the epidemiology and distribution of arthropod-borne viruses. In a study on spring-summer encephalitis in Russia, Pavlovsky 1940, (in Arthur, 1962) states that naturally infected I. persulcatus ticks are abundant in the first half of the summer, but virus is not identifiable in those collected in the latter part of July and August. It is indicated that virus may be inactivated during these months as result of some environmental factor. Sooter (1959) suggested that weather conditions favoring the vector population during key spring months are necessary before outbreaks of arthropod-borne encephalitis can occur in the U.S.A., and that study of weather data might make possible the prediction of outbreaks. Reeves and Hammon (1962b) reported that in Kern County, California, western encephalitis virus was first detected in Culex tarsalis during or following half-month periods when mean daily temperatures rose above 85°F, but St. Louis (SLE) virus was usually detected only after temperatures rose above 85°F for a few days or weeks.

In Egypt the lowest temperatures occur in December and January, the highest in July and August. Assuming that the ticks do not feed from late October until late February, the spring peak in the graph (figs. I and II) may represent activation of the virus obtained the previous year, owing to warmer weather and not to a new crop of birds. Also, the blood meal from the newly arriving adult birds might play a role in boosting this activity (Philip, 1958). By the time the bulk of the tick population are ready for a second meal (after 25-50 days), most of the nestlings would probably have developed a partial age resistance to Quaranfil infection (Sudia and Chamberlain, 1959). These that are fed upon shortly after hatching might die of infection with Quaranfil virus, as is the case with baby chicks, unless protected by maternal antibody (Sooter et al., Reeves et al., Kissling et al., 1954). A short-lived viremia tends to restrict the number of nymphal and adult ticks to become infected. The host reaction against more than approximately 20 larvae engorging to repletion would tend to restrict infection of this stage also, as well as a too overwhelming increase in the tick population. The low infection rate with Quaranfil virus maintained during summer months may also be due to the build-up of an immune bird population, and to the inability of the virus to pass transovarially from the adult tick to its progeny.

Contrary to findings with Quaranfil, Nyamanini virus infection was maintained at the spring high level in ticks all through the summer months. The fact that in experimental studies this virus did not kill or cause detectable viremia in baby chicks suggests that

infection may be mild in young herons also, and might stimulate a lesser antibody response, permitting continual transmission to occur throughout the summer. A high viremia is indication of a quick and massive multiplication of virus which in turn is strongly antigenic and stimulates high antibody production. A lesser viremia may result in an immunity of a low order. For example, with eastern equine ence-phalitis virus in rabbits, a refractory animal to this virus, several inoculations of the virus has to be administered to obtain an anti-serum of a reasonably high titer (Chamberlain, personal communication). Clarification of the Nyamanini host relations must await further field studies to determine natural antibody levels in the heron population. It may even be possible that Nyamanini is perpetuated transovarially.

The fall peaks of tick infection with the two viruses probably represent an increase of the virus through involvement of birds which hatched toward the end of the nesting season. These late nestlings are usually much fewer in number than the spring crop, smaller and weaker, but may play a major role in building up the infected tick pool for carrying the viruses from one year to the next.

In all, the factors discussed contribute to a well-balanced relationship between the bird and tick populations on one hand and the propagation of the virus on the other. The cycle of Quaranfil virus as indicated by these studies represents one of the simplest known for arboviruses since the arthropod itself is the obvious reservoir. Although Quaranfil virus is apparently essentially a bird virus, the acarine vector sustained by and restricted to the avian population

provides for its maintenance and dissemination.

V. SUMMARY AND CONCLUSIONS

1. *Argas* ticks from the heron rookeries in Egypt, Tanganyika, and South Africa are a distinctive species that differs morphologically, biologically, ecologically and in the organisms it harbors from the cosmopolitan fowl tick, *A. persicus*. Consequently, the new species, *A. arboreus*, was described, and the new subgenus, *Persicargas*, was erected to include *arboreus*, *persicus*, and other related species.

2. The life-cycle of *A. arboreus* under insectary conditions of 27°C and 75% R.H. requires about 3 months. In averages, females oviposit 6 days following feeding; LL hatch after 16 days, feed for 6 days and molt to N_1 7 days after repletion and dropping off the host; N_1 molts to N_2 7 days after feeding; moltings of N_2 to N_3 or adult, or of N_3 to adult occur 14 days following feedings. Considering that the life cycle of the tick is longer under natural conditions, probably two generations of the tick are produced from March until October, the nesting period of the bird host, *Bubulcus i. ibis*.

3. *A. arboreus* ticks sampled from the Delta Barrage area of Egypt were commonly infected with two viruses, Quaranfil and Nyamanini. The Egyptian records of Nyamanini virus reported here represent the first findings outside South Africa, where it had earlier been

isolated and reported.

4. During the study period, the natural infection indexes of *A. arboreus* with each of Quaranfil and Nyamanini viruses showed an early spring rise and a less intensive rise during the fall (figures I and II). Although the spring rise of Nyamanini was maintained during the spring and most of the summer months, that of Quaranfil dropped in mid-spring and remained at a low level until the fall.

5. The percentages of pools of *A. arboreus* ticks infected with Quaranfil and Nyamanini viruses were 8.4% and 15.2% respectively, with each pool consisting of 5 ticks. The possibility of concurrent natural infection of single ticks with both viruses appeared unlikely but on three occasions both viruses were isolated from the same tick pools.

6. The probable presence of Quaranfil and Nyamanini virus antibodies in the residual blood contained by field-collected *A. arboreus* apparently had little or no diminishing effect upon detection of viruses when the ticks were ground and tested. Also, experimentally given immune blood meals did not have an apparent adverse effect on detection of Quaranfil virus in ticks incubated 10 days following the infective meal before grinding.

7. *A. arboreus* ticks were found infected with Quaranfil and Nyamanini viruses the year around, proving this species to be an efficient

reservoir for both.

8. No viruses were isolated from _A. persicus_ from three localities in Egypt.

9. Quaranfil and Nyamanini viruses were found to be antigenically unrelated to each other or to Chenuda virus. Also they were shown to differ in their pathogenicity to suckling and 3-week-old mice, baby chicks, chickens and adult herons. Although both viruses formed plaques in duck embryo monolayer tissue culture, they differed in their behaviour and cytopathogenic effect.

10. Quaranfil virus produced viremia in baby chicks. The highest viremia levels were reached on the fourth and fifth days following the virus inoculation.

11. Both viruses were unable to hemagglutinate goose red cells at a pH range of 5.7 to 6.8.

12. Experimental transmission studies of Quaranfil virus by _A. arboreus_ indicated a high vector potential of this species. Evidence is presented that a widespread infection beyond the digestive tract of the tick takes place by and after 44 days of extrinsic incubation.

13. _A. persicus_ ticks can be experimentally infected with Quaranfil virus and can retain the virus for long periods (120 days). However, transmission was accomplished only by mass feeding,

indicating the species to be a poor vector. Since the virus was detected in only 4% of the coxal fluids examined, suggesting poor spread beyond the digestive tract, it seems possible that mechanical transmission through regurgitation of partly digested blood still containing some active virus might have accounted for the transmissions noted. The importance of A. persicus as a vector of Quaranfil virus in nature would appear to be minimal.

14. Transtadial transmission of Quaranfil virus was obtained with both A. arboreus and A. persicus ticks. However, it is not considered as good a measure of vector potentiality as transmission by bite.

15. The failure to demonstrate transovarial transmission of Quaranfil virus with A. arboreus, the natural vector, indicates that a vertebrate host is necessary for its perpetuation. Undoubtedly, Quaranfil is a bird virus which is transmitted in nature by the vector tick.

16. Transovarial transmission of Quaranfil virus by A. persicus also could not be demonstrated.

17. A. arboreus ticks were found relatively refractory to experimental infection with the mosquito-borne virus of St. Louis encephalitis. Those few ticks that acquired the infection were able to maintain the virus at a high titer for a period in excess of 52 days but did not transmit by bite. The efficiency of transtadial transmission from one nymphal stage to the next and to the adult stage

was poor. Transovarial transmission was not obtained. The long retention of the virus does suggest the possibility of some members of the Argas complex serving as SLE reservoirs, however, despite the poor evidence of transmissibility, in view of the close association of this tick group with bird populations.

18. Experimental infection with Quaranfil virus did not have an adverse effect on the ticks, their longevity or their parasitic periods. Long survival of field-collected ticks in the laboratory, despite natural infections with Nyamanini virus, indicates this to be true for this virus also.

19. The attempts to transmit Quaranfil virus infection to baby chicks via the oral route with negative results suggest that transmission by nest contamination is unlikely to occur in nature.

20. It is concluded that differences in susceptibility to Quaranfil virus in *A. arboreus* and *A. persicus*, genetically controlled, account for the vector role of the one species and its lack in the other, rather than ecological factors. The latter comes second in the host-parasite relationship; although not determining in itself, it may be limiting.

21. The present investigation has indicated fruitful fields for further study.

    a. To interpret fully the seasonal incidence of the two viruses

in the *A. arboreus* ticks, correlations between infections in the cattle egrets (both adult and nestling) and the ticks are necessary. Other studies on the population dynamics of the ticks and birds would be significant.

b. A study on possible interaction or interference between Quaranfil and Nyamanini viruses would be of interest, using the invertebrate vector, *A. arboreus*, and the vertebrate host, the cattle egret.

c. Attempts to establish deep-seated virus infections in the tick larval stage, before possible ovarian barriers have developed (Bertram *et al*., 1962), might result in successful transovarian transmission by subsequent adults.

d. Experimental studies should be conducted to determine if a spring blood meal "activates" overwintering virus in the host tick.

e. Experimental infection and transmission studies should be conducted with Nyamanini virus, using *A. arboreus* and adult and nestling cattle egrets. The relatively high level of tick infection throughout the summer months suggests either continued transmission without depletion of the vertebrate host population, or transovarial transmission. These points should be checked.

f. The incidence of maternal antibody in both early and late

broods of nestlings, and its effects on the infection course in the birds and on the tick infection rate should be checked.

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PLATES

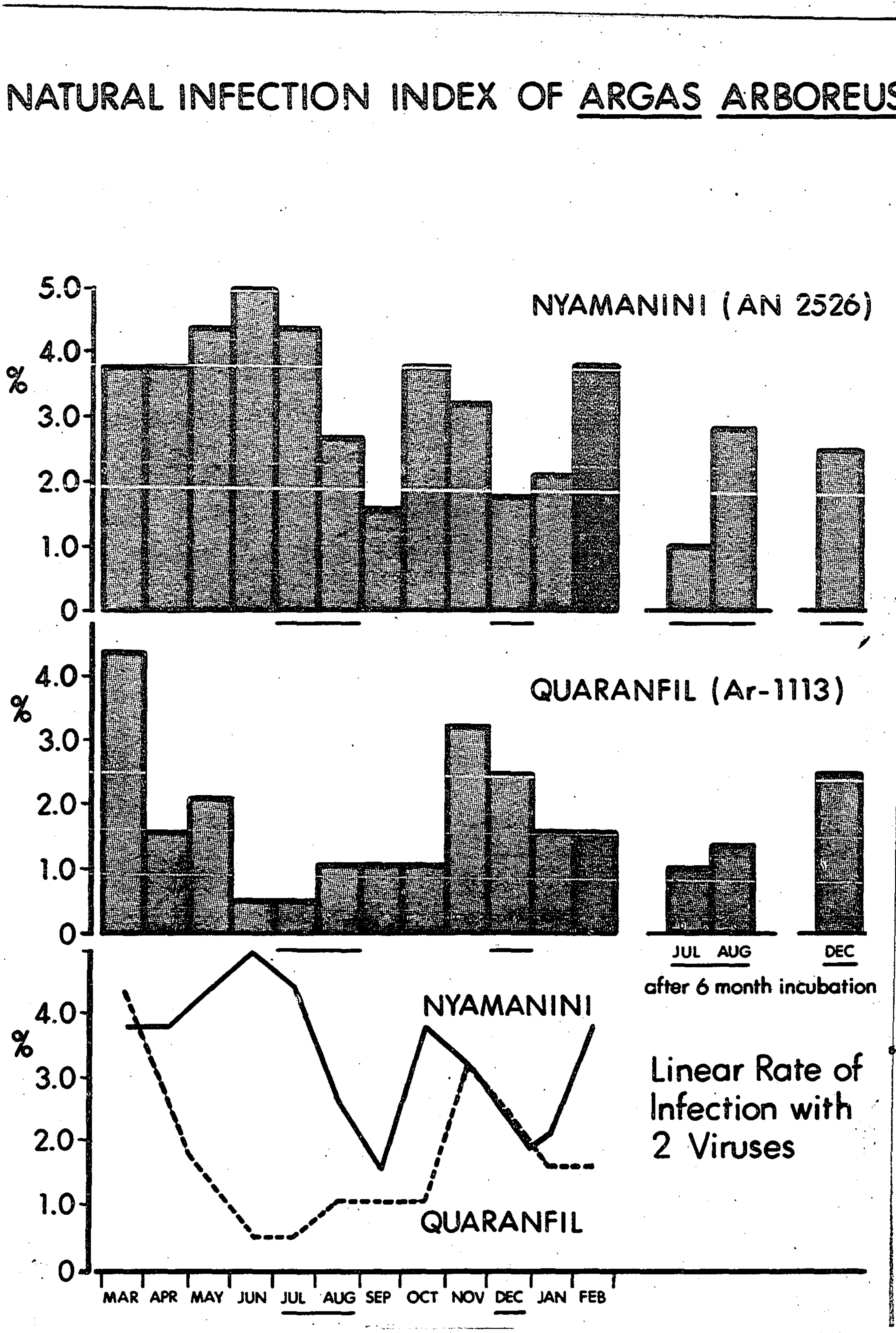
NATURAL INFECTION INDEX OF ARGAS ARBOREUS
NYAMANINI (AN 2526)
QUARANFIL (Ar-1113)
%
5.0
4.0
3.0
2.0
1.0
0
JUL AUG
DEC
after 6 month incubation
NYAMANINI
QUARANFIL
Linear Rate of Infection with 2 Viruses
MAR APR MAY JUN JUL AUG SEP OCT NOV DEC JAN FEB

Fig. 1

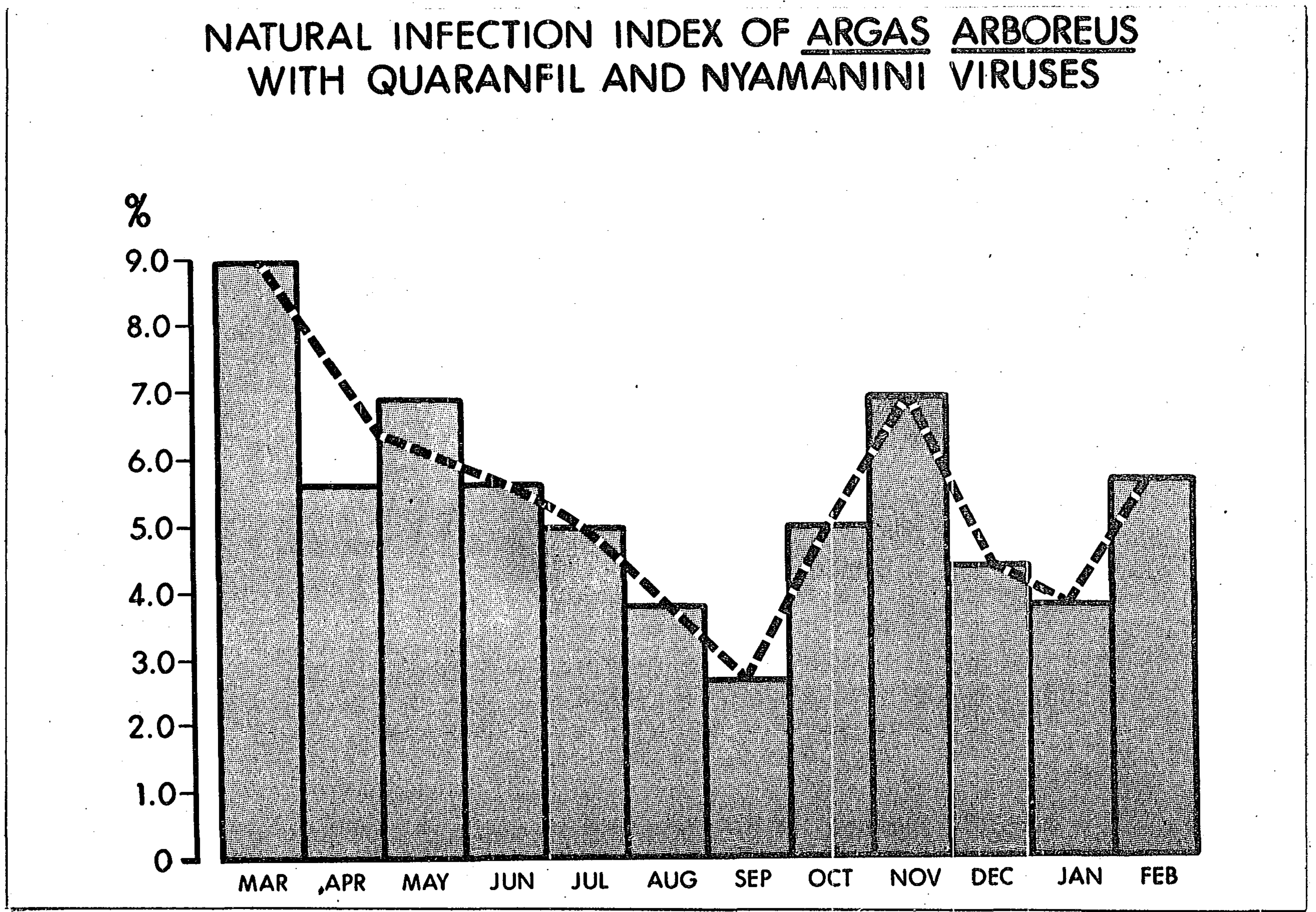
NATURAL INFECTION INDEX OF ARGAS ARBOREUS
WITH QUARANFIL AND NYAMANINI VIRUSES
%
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
MAR
APR
MAY
JUN
JUL
AUG
SEP
OCT
NOV
DEC
JAN
FEB

Fig. 2

FIG. 3: IDENTIFICATION AND SORTING OF TICKS

Monthly field collections are identified, then sorted according to sex and nymphal stage. Later, ticks are assorted into pools in appropriately labeled Kahn tubes.

FIG. 4: PROCESSING TICKS FOR INOCULATION

A drop of diluent and a small amount of powdered Alundum is added to each frozen tick pool in a mortar and the ticks are thoroughly ground. Two ml of diluent are then added to each mortar and the grinding continued to make an even suspension.

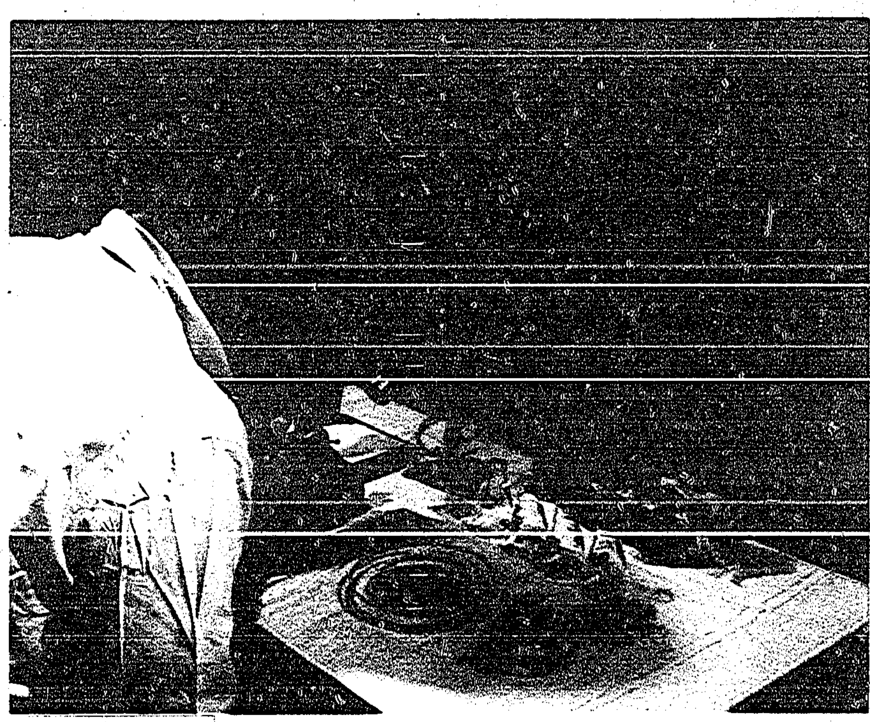

Fig. 3

Fig. 4

FIG. 5: INOCULATION OF TICK SUSPENSIONS

Suckling mice are each inoculated intracerebrally with 0.02 ml of the tick suspension. It is important to introduce the needle vertically into the brain case at either side of the sagital suture to reduce trauma.

FIG. 6: SIGNS OF VIRUS INFECTION

A litter is showing typical signs of virus infection. Dead, prostrate and convulsing baby mice are scattered about and there is no nest. The mother is behaving erratically.

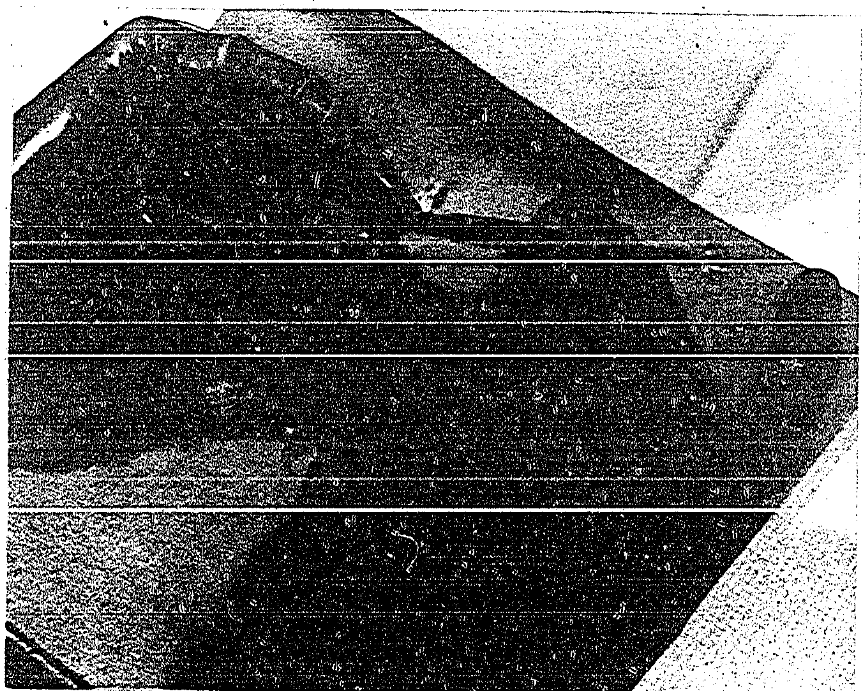

Fig. 5

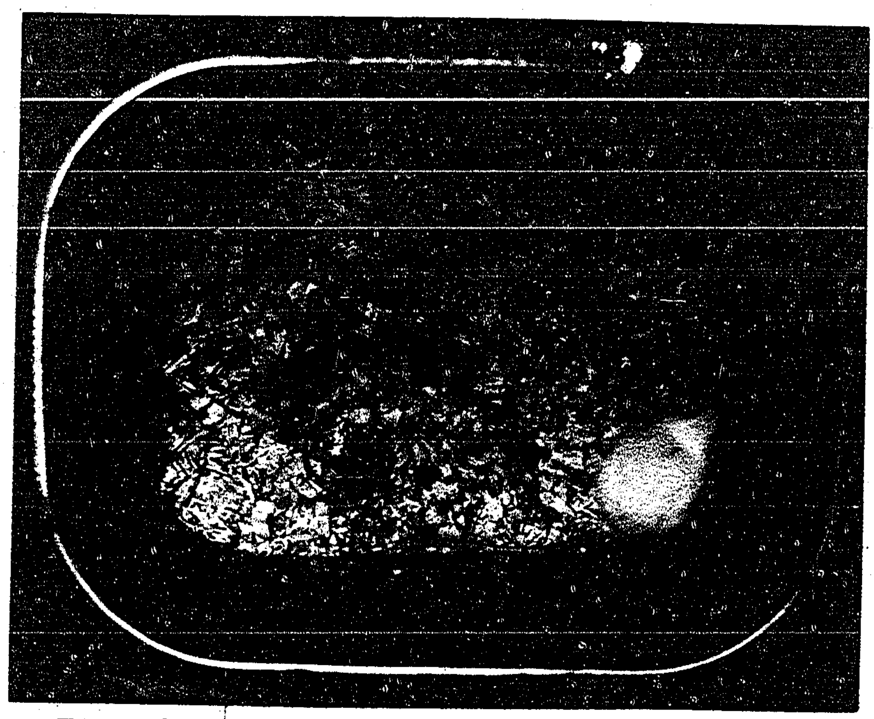

Fig. 6

FIG. 7: HARVESTING OF MOUSE BRAINS

Infected brains are aspirated directly from the unopened skulls of thawed mice by means of a large 15-gauge needle attached to a vacuum system. A flask containing 50% Chlorox is used as a safety trap before the vacuum line. The receptor is kept in a dry ice and alcohol bath.

FIG. 8: REMOVAL OF ACETONE BY ASPIRATION

This vacuum system is utilized for aspiration of acetone during the preparation of sucrose-acetone extracted antigens. Two large flasks consecutively connected to a water pump and partly submerged in boiling water collect the aspirated fluid.

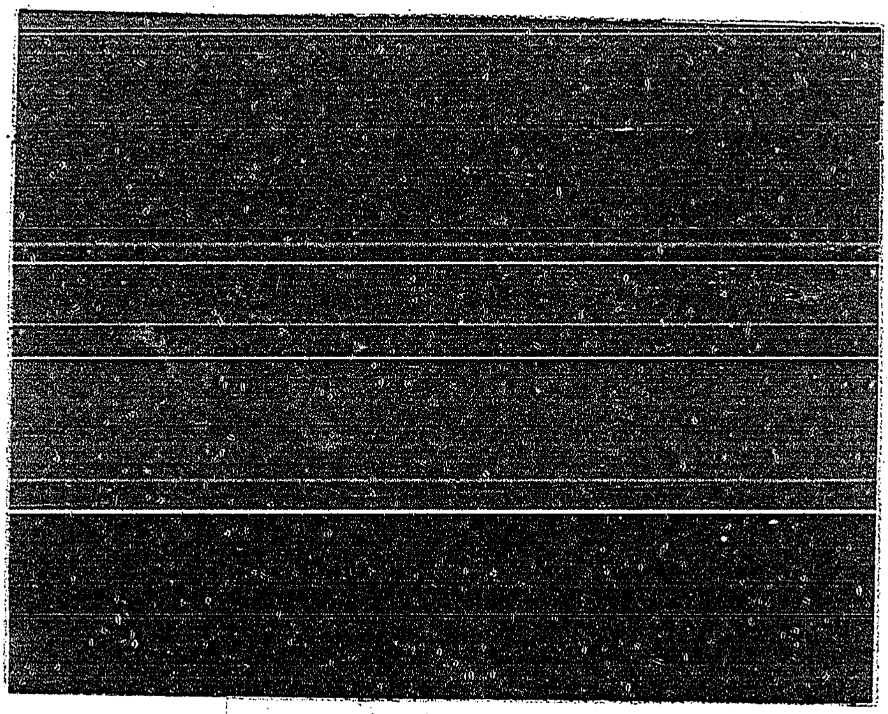

Fig. 7

Fig. 8

FIG. 9: VACUUM-DRYING OF ANTIGEN

This system is used for vacuum-drying of sucrose-acetone extracted antigen. To remove the antigen after drying the rubber tubing between the last flask and the glass Y tube and the tubing on the plugged arm of the Y tube are clamped off. The tube plug is then removed and the Y arm clamp is released very slowly, allowing the air to enter gently so as to minimize stirring of antigen powder. After switching off the pump the remaining clamp is released slowly allowing air to bubble in the Clorox solution in the middle flask. Then, under a hood, the Y tube with the centrifuge bottles containing antigens is disconnected and antigen bottles fitted with solid plugs.

FIG. 10: LYOPHILIZATION OF ANTIGEN

Sucrose-acetone antigen is lyophilized overnight. Before removing the ampules, the pump is switched off and the pressure equalized with nitrogen gas.

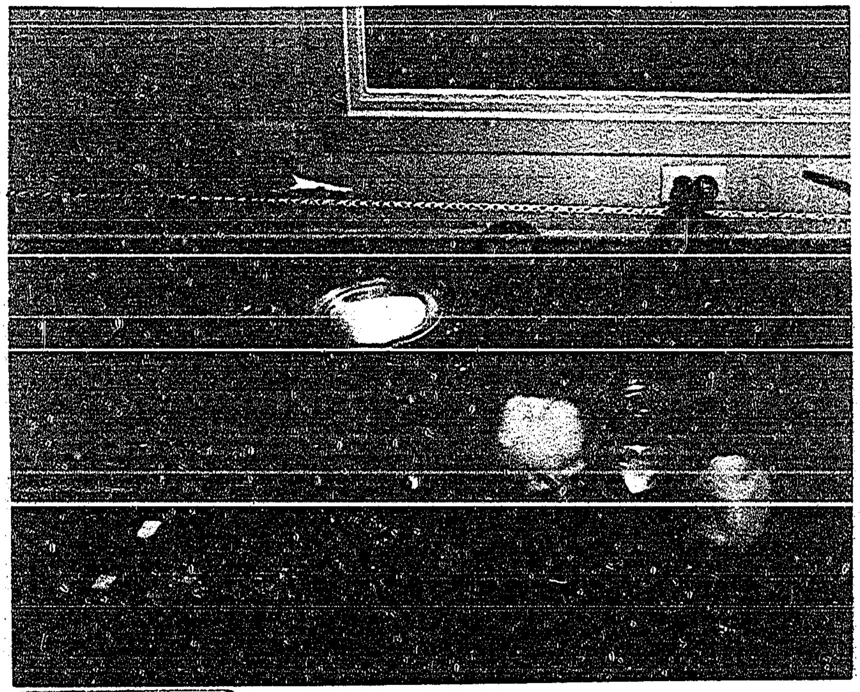

Fig. 9

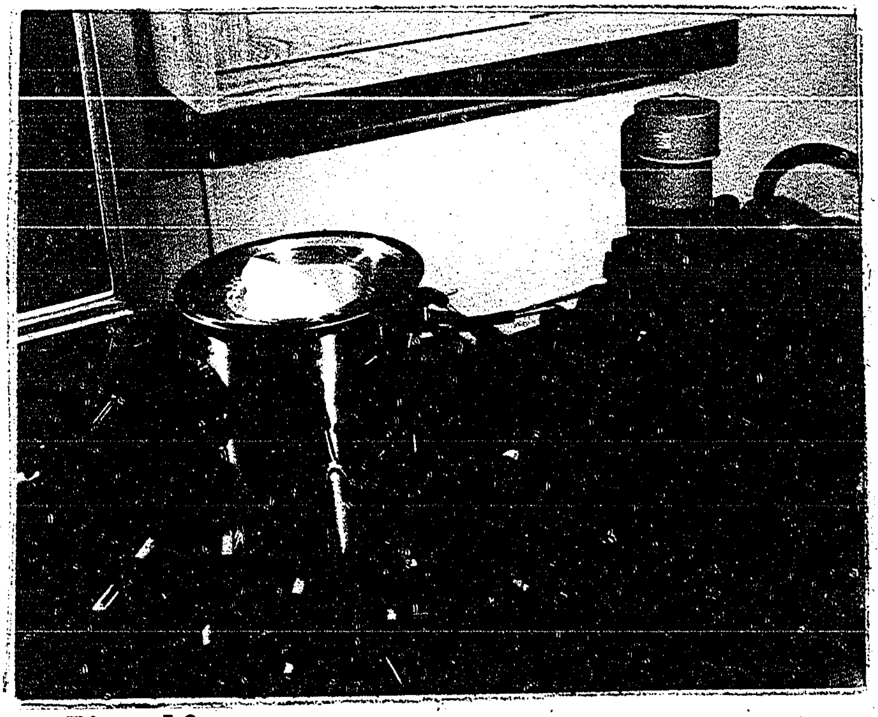

Fig. 10

FIG. 11: COLLECTION OF HYPERIMMUNE SERUM

Each mouse is bled from the heart for hyperimmune serum. Prior to bleeding it was anesthetized by brief exposure to ether and the bleeding area swabbed with 70% alcohol. The tail is held taut between the little and ring fingers.

FIG. 12: READING THE MICRO-CF TEST

Results of the CF test performed by the microtiter system are read with the aid of a reflector mirror. Aluminum plate-carriers are used for centrifugation of plates prior to reading.

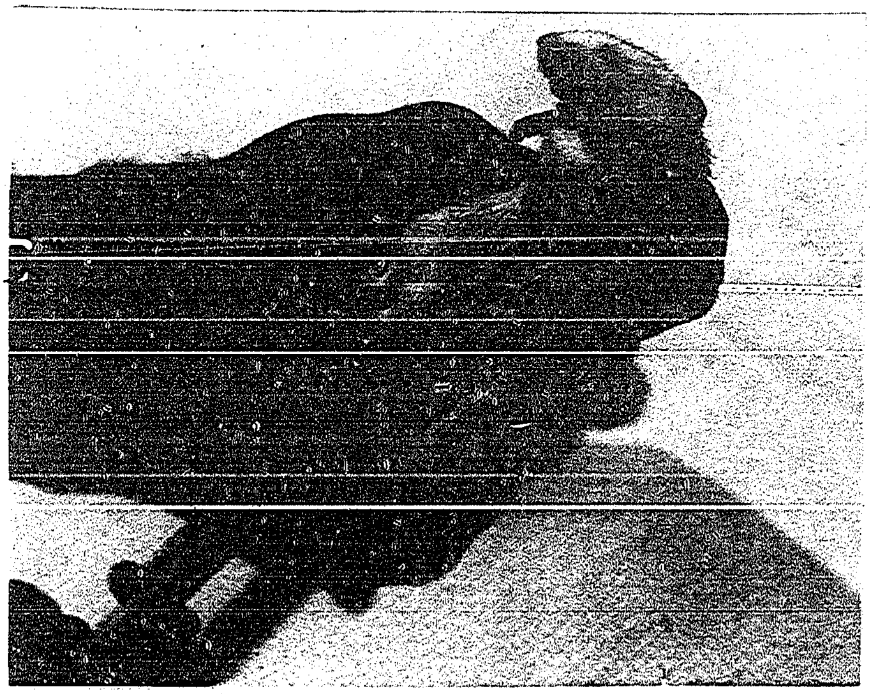

Fig. 11

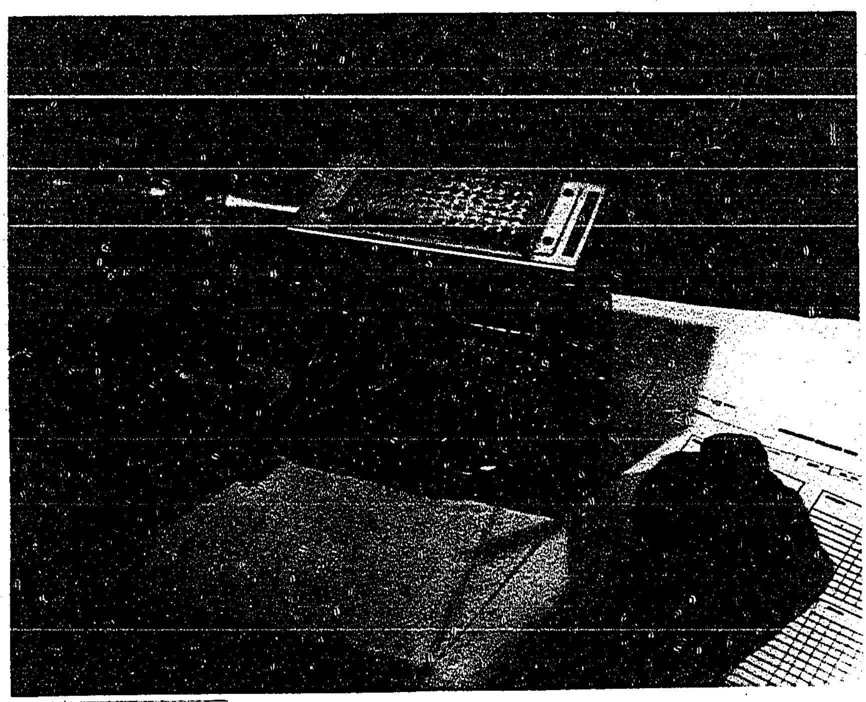

Fig. 12

FIGS. 13 & 14: CF MICROTITER TEST

Crude brain antigens Nos. 49 through 72, each representing a field isolation, are tested by the micro-titer system against Quaranfil hyperimmune mouse serum. Serum dilutions are 1:32, 1:64, 1:128 and 1:256. Antigens Nos. 51, 53, 56, 57, 60-63, 67, 68, 74-76, 83 and 89-94 have fixed complement in the presence of the antiserum, showing them positive for Quaranfil virus. The numerical values of the degree of fixation obtained with antigen 61 and the highest to lowest dilution of antiserum are 4, 3, 2 and 0, respectively.

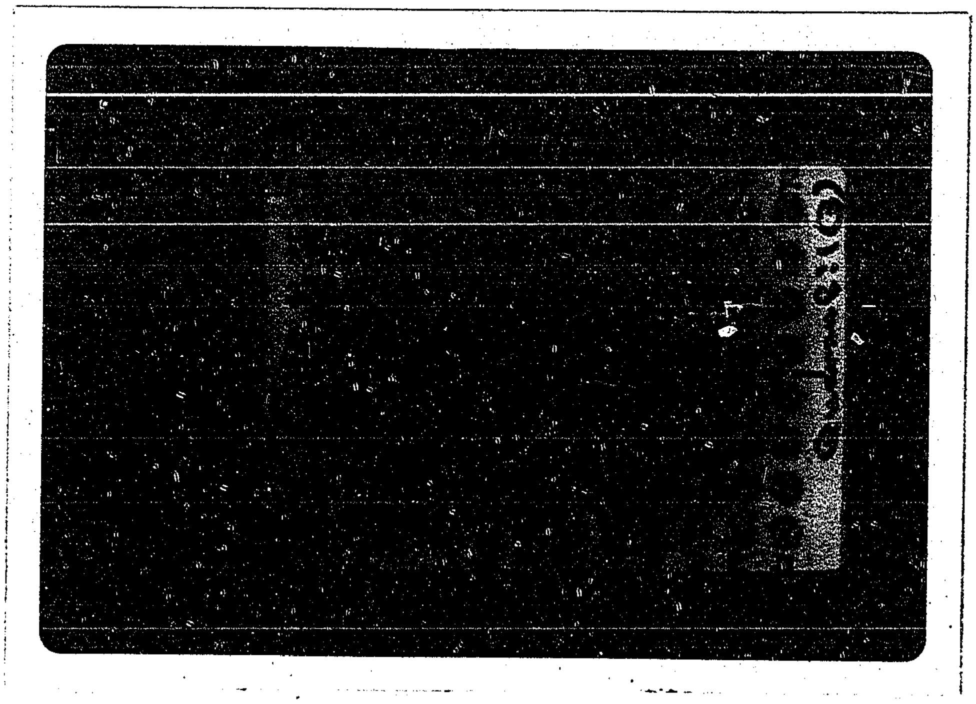

Fig. 13

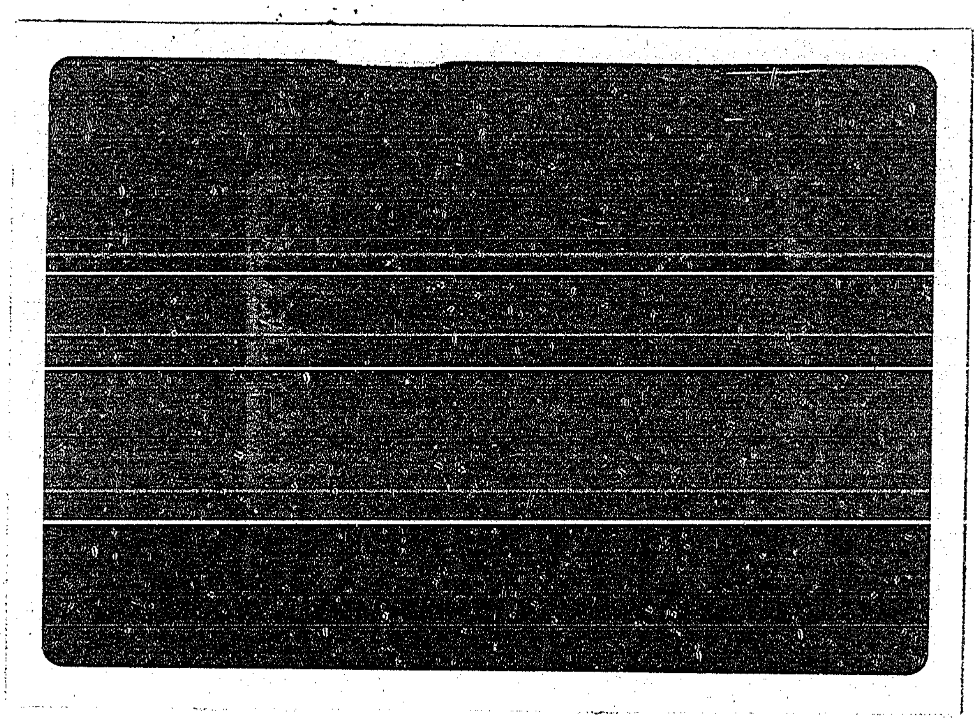

Fig. 14

FIG. 15: TICK REARING EQUIPMENT

The tick rearing room is equipped with moated tables, rabbit cages, and a tick rearing shelf.

FIG. 16: TICK FEEDING CAPSULE

The tick feeding capsule is placed on a pigeon with an adhesive girdle. A pad of polyurethane foam provides a tight seal between the pigeon's body and the capsule.

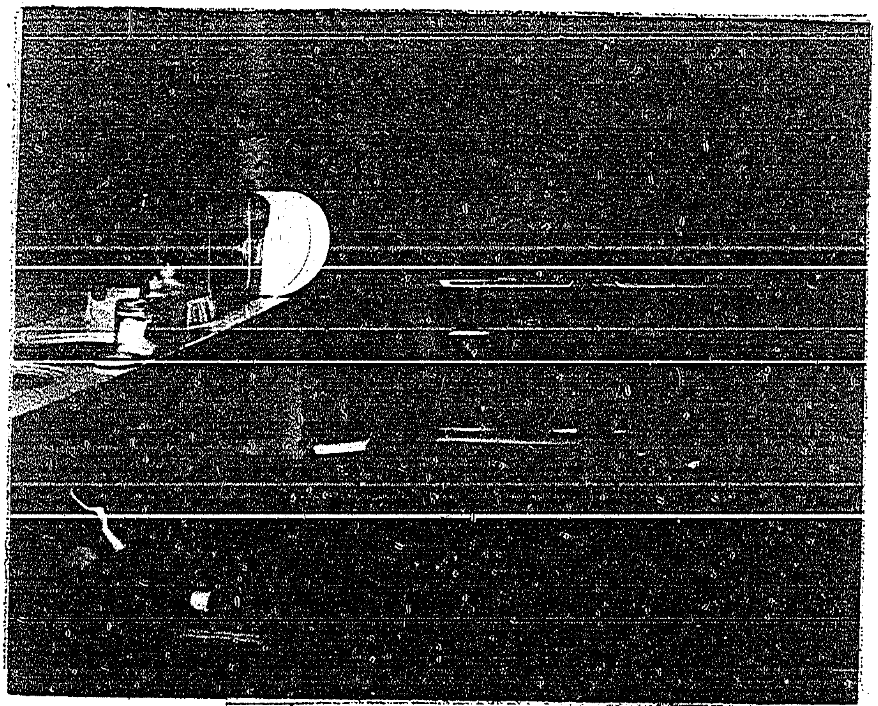

Fig. 15

Fig. 16

FIG. 17: SYMPTOMS OF VIRUS INFECTION IN CHICKS

Chicks are showing typical symptoms of infection with Quaranfil virus, i.e. weakness, sleepiness and death. A chick usually dies within few hours after onset of symptoms.

FIG. 18: VIRUS TRANSMISSION WITH ARGAS TICKS

Ticks are given the initial infected meal in the transmission study with Quaranfil virus. They are fed upon viremic chicks prostrate with the infection.

Fig. 17

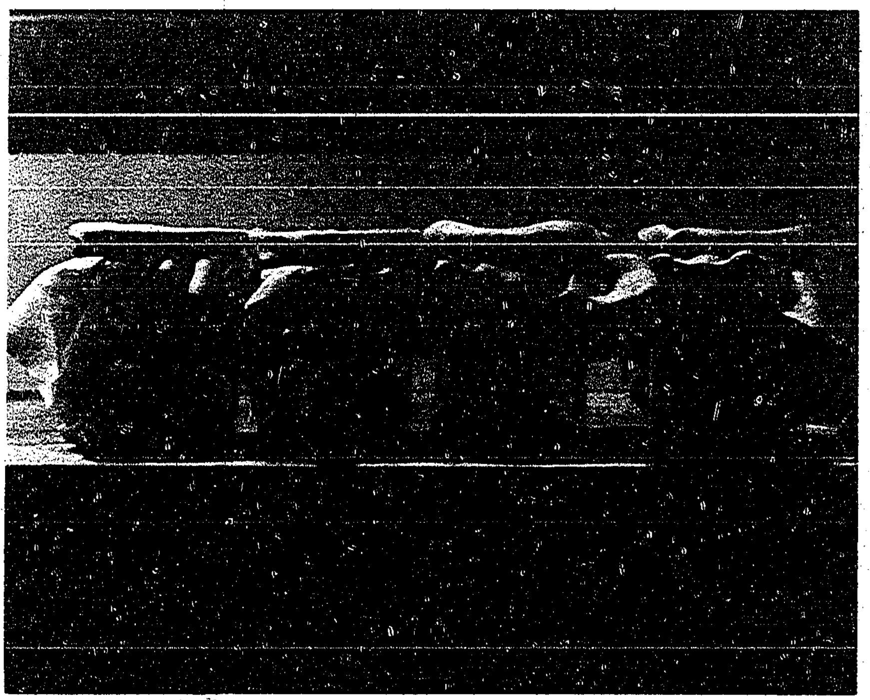

Fig. 18

FIG. 19: CYTOPATHIC EFFECT OF VIRUSES IN PEKIN DUCK EMBRYO MONOLAYER TISSUE CULTURE*

*Camera: 4"x5" Linhof, 150 mm lens, full bellows extention.

Film: 4"x5" Contrast Process Panchromatic, developed in D-76 at 68°F for 8 min.

Illumination: Dark field.

QUARANFIL NYAMANINI CHENUDA

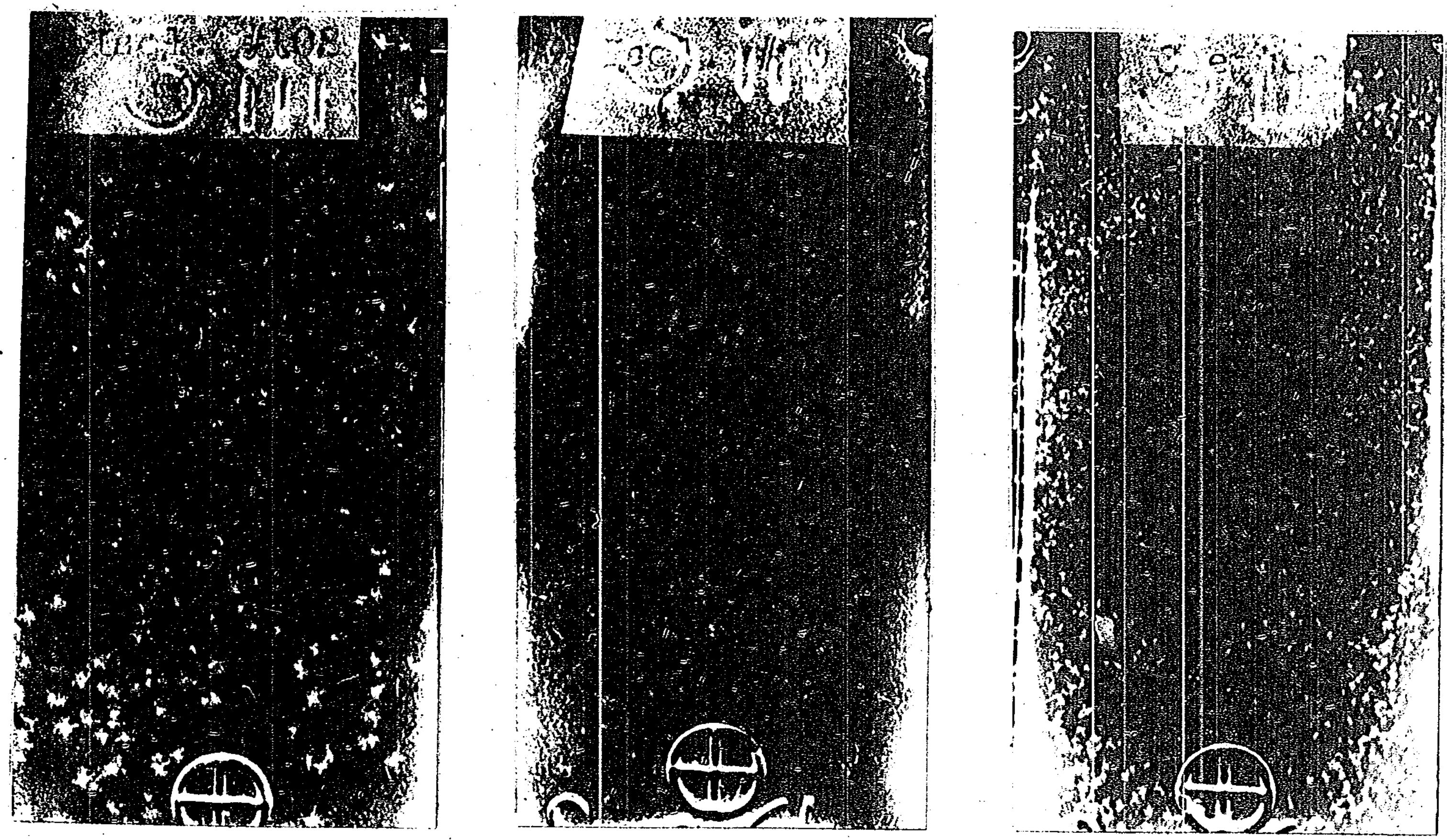

Fig. 19

TABLES

TABLE I

LIFE CYCLE OF ARGAS ARBOREUS,
REARED AT 27°C and 75% R.H.

Tick No.	Successive Hosts and Days to Lay Eggs	Days to Hatch	LL, Days Feeding (No. LL)	LL Molt to N_1 Days (No. N_1)	N_1 Molt to N_2 Days (No. N_2)	N_2 Molt to Adult Days (No. A)	Adults ♂♂	♀♀	N_2 Molt to N_3 Days (No. N_3)	N_3 Molt to A Days (No. A)	Adults ♂♂	♀♀
♀14	C* 50	16	7(7)	7(6)	10(1)							
	P* 5	17	8(3)	7(1), 8(1), 12(1), 14(1)	17(2)				13(1)	13(1)		1
	P 4	17	10(12)	6(4), 8(1), 12(6)	12(2), 16(2), 23(1), 30(1), 35(1)				9(1), 13(2)	15(3)		3
	P 8	18	12(11)	7(5), 8(3), 10(2)	14(1), 16(1), 18(1), 24(1)							
			6(5)	7(1), 9(1)	12(2)							
			7(2)	6(1)								
			5(15)	7(14)	13(14)	14(9), 26(1)	5	5	14(1)	14(1), 16(3)		
			8(4)									
♀15	C 35	14	8(1)	9(1)	22(1)				13(1)		1	
	P 8	15										
♀16	C 32											
	P 3	15	8(6)	8(2)								
	P 5	17										
♀17	P 5	15										
	P 6	15										
♀18	C 19	14	6(3), 7(2), 14(2)	8(4), 9(1)	10(1), 11(1), 17(1), 20(1)	13(1), 14(1)	2					
	P 4	16	5(12)	6(3), 7(8)	10(3), 11(8)	13(8), 16(3)	5	6				
	P 10	17										
♀19	C 66	18										
	P 10	16										
♀20	C 10	16										
	P 12											
	P 5	16	9(13)	8(1), 10(3), 12(8)	9(1), 27(2)	13(1)	1		13(2)	19(2)		2
♀21	C 46	19	4(1)	9(1)	11(1)							
			5(3)	8(2)	12(1)	16(1)		1				
			6(3)	7(2), 11(1)	8(1)							
			8(4)	5(1), 7(1), 9(2)	18(2)				16(1)	19(1)	1	
	P 4	17										
	P 8	12										
♀22	C 20	17	6(6)	9(6)	11(1), 12(1), 13(2), 18(1)	11(1), 12(1), 13(1), 14(1)	2	2				
			7(3)	7(3)	11(1), 12(2)	17(1)	1		16(1), 18(1)	14(1), 15(1)		2
♀23	C 56	16	6(4)	8(2)	8(1), 12(1)	12(1), 13(1)						
	P 4	18	8(4)	7(3)	9(2)	15(1), 16(1)						
	P 7	13										
♀24	C 75	13										

			6(3)	7(2), 11(1)	8(1)							
			8(4)	5(1), 7(1), 9(2)	18(2)				16(1)	19(1)	1	
	P 4	17										
	P 8	12										
♀22	C 20	17	6(6)	9(6)	11(1), 12(1), 13(2), 18(1)	11(1), 12(1), 13(1), 14(1)	2	2				
			7(3)	7(3)	11(1), 12(2)	17(1)	1		16(1), 18(1)	14(1), 15(1)		2
♀23	C 56	16	6(4)	8(2)	8(1), 12(1)	12(1), 13(1)						
	P 4	18	8(4)	7(3)	9(2)	15(1), 16(1)						
	P 7	13										
♀24	C 75	13										
	P 6	15										
♀25	C 68	14										
	P 4	17	4(30)	7(9), 8(21)	10(6), 11(20)	12(25)	14	11	12(1)	15(1)		1
			5(12)	6(9), 7(3)	7(8)	14(8), 24(1)	6	3	14(2)	16(2)		2
			7(4)	8(4)	12(3)	18(2)	2		16(1)	20(1)		1
			8(2)	7(1), 8(1)								
			5(21)	5(21)	18(19)	14(4), 23(1)	4		20(4)	19(4)	1	3
	P 4	13	7(21)									
	P 5	17										
	P 5											
♀26	P 3	16	7(8)	7(8)	13(4)	17(2)	2		16(2)	18(1)	1	
	P 5	11										
	P 5	14										
♀27	C 72	15	5(28)	6(4), 7(24)	10(6), 11(22)	10(2), 12(9), 14(7)	10	3	11(1), 14(5)	13(8)		8
			11(2)	7(2)		13(1)	1					
	P 7	13	8(10)									
	P 7	15										
♀28	C 64	15	7(2)									
	P 5	16										
♀29			7(1)	13(1)	11(1)	16(1)	1					
♀48		15	5(4)	6(4)	7(1), 11(1), 13(1)	16(1)	1			13(1)	1	
			6(8)	5(8)	10(5), 12(3)	13(2), 14(3)	3	2	11(1), 13(1), 16(1)	13(3)		3
			7(7)	5(7)	10(4), 12(3)	13(3), 14(3)	4	2	12(1)	14(1)		1
			9(13)	4(13)	10(3), 12(9)	12(2), 13(3), 14(2)	6	1	11(3), 12(2)	13(1), 14(3), 16(1)		5
			11(4)	7(4)	10(2), 12(1)	12(1)	1		13(2)	17(1)		1
♀50		16	7(20)	10(20)	13(7)	18(5)	4	1	16(7)	17(5)	2	4
	P 5	17										
	P 8	20										
♀53	P 6	17	7(1), 9(2)	8(1), 9(2)	14(2)	16(2)	2					
	P 5	15										
♀52	P 11	15										
	P 6	17										
Median	5 **	16	7	7	11	13			14	15		
Mean	6.0**	15.7	6.7	7.4	12.7	13.8			14.6	15.5		

* C = 1-day-old chicks; P = Adult pigeons.

** Median and mean of days required for egg laying after feeding on pigeons only. The comparable values following the first feedings on chicks are 50 and 47.2 days respectively.

TABLE II

ADSORPTION OF QUARANFIL VIRUS
IN MONOLAYER DUCK EMBRYO TISSUE CULTURE

CONTROL

VIRUS DILUTION	NCS		CS 60^{1st}		CS 58^{1st}		CS 60^{1st}/CS 58^{1st}	
	Max. No. of Plaq. (Day)	Log PFU/ML.	Max. No. of Plaq. (Days)	Log PFU/ML.	Max. No. of Plaq. (Day)	Log PFU/ML	Max. No. of Plaq. (Day)	Log PFU/ML.
Quar. 10^{-2}	120 (D8)	2.4	0	-	171 (D8)	2.5		
10^{-3}	21 (D8)	1.6	0	-	30 (D8)	1.8		
10^{-4}	3 (D8)	0.8	0	-	1 (D8)	0.3		
10^{-5}	0	-	0	-	1 (D8)	0.3		
10^{-6}	0	-	0	-	0	-		
St.60 10^{-2}	TNC (D6)	?	0	-	TNC (D6)	?		
10^{-3}	172 (D8)	2.5	0	-	261 (D8)	2.7		
10^{-4}	16 (D8)	1.5	0	-	45 (D8)	2.0		
10^{-5}	5 (D8)	1.0	0	-	8 (D8)	1.2		
10^{-6}	1 (D8)	0.3	0	-	0	-		
Nyam. 10^{-2}	349 (D11)	2.8	357 (D11)	5.9	2 (D11)	0.6		
10^{-3}	153 (D11)	2.5	56 (D11)	6.1	0	-		
10^{-4}	17 (D11)	1.5	13 (D11)	6.4	0	-		
10^{-5}	6 (D11)	1.1	1 (D11)	6.3	0	-		
10^{-6}	0	-	0	-	0	-		
St. 58 10^{-2}	>400 (D8)	>2.9	154 (D8)	5.5	0	-		
10^{-3}	76 (D8)	2.2	56 (D8)	6.1	0	-		
10^{-4}	5 (D11)	1.0	5 (D11)	6.0	0	-		
10^{-5}	1 (D11)	0.3	0	-	0	-		
10^{-6}	0	-	0	-	0	-		
St.58/St.60 10^{-2}	TNC (D6)	?	139 (D8)	5.4	TNC (D6)	?	2 (D11)	0.6
10^{-3}	86 (D8)	2.2	19 (D11)	5.6	170 (D6)	2.5	0	-
10^{-4}	29 (D11)	1.8	2 (D11)	5.6	14 (D8)	1.5	0	-
10^{-5}	2 (D11)	0.6	0	-	5 (D11)	1.0	0	-
10^{-6}	0	-	0	-	0	-	0	-

TEST

*A total of 42 virus isolates (tick suspensions) identified by CF tests as Quaranfil were tested by the adsorption method in attempt to detect mixed infection with Nyamanini virus. The three listed here were proven to be mixed infections by plaque selection and passage in mice.

Tick Susp. Positive After Adsorp.*	CS 60^{1st} Max. No. of Plaq. (Days)
Ap-11A ♂ (26-30)	1 (D11)
♀ (26-30)	3 (D11)
N2 (11-15)	4 (D11)

NCS = Normal chicken serum
CS 60^{1st} = Immune chicken serum against stock 2560-61; first bleeding, 1 month after immunizing inoculation
CS 58^{1st} = Immune chicken serum against stock 2558-59; first bleeding, 1 month after immunizing inoculation
PFU/ML = Number of plaque forming units per ml. of diluted virus stock
Quar. = Quaranfil virus
St. 60 = stock 2550-61
Nyam. = Nyamanini virus
St. 58 = Stock 2558-59
TNC = Too numerous to count

TABLE III

NEUTRALIZATION TESTS IN DUCK-EMBRYO MONOLAYER TISSUE CULTURE; NUMBER OF VIRUS PLAQUE-FORMING UNITS PER ML LOG_{10}

VIRUS or SM BRAIN ISOLATE	NORMAL SERA		HETEROLOGOUS SERA				HOMOLOGOUS SERA			
	NMS	NCS	MS Ny	MS 58	CS 58^{1st}	MS Ch	MS Qu	MS 60	CS 60^{1st}	CS 60^{2nd}
Quar.	5.1	5.4	5.2	5.0	5.1	4.8	2.3	2.3	0	
St. 60	6.2	6.4	6.3	6.1	5.9	5.6	0	0	0	0
$3AN_1$ (1-5)	>7.3	>7.3	>7.3	>7.3	7.3	7.0	2.3	3.3	2.3	
11A ♂ (21-25)	>7.3	>7.3	>7.3	7.1	7.0	7.0	3.3	4.2	0	
8A ♂ (55-60)	7.0	7.0	6.9	6.7	6.8	6.4	3.0	4.0	0	
$21AN_1$ (26-30)	>7.3	>7.3	>7.3	>7.3	>7.3	>7.3	3.0	4.1	0	

VIRUS or SM BRAIN ISOLATE	NORMAL SERA		HETEROLOGOUS SERA				HOMOLOGOUS SERA			
	NMS	NCS	MS Qu	MS 60	CS 60^{1st}	MS Ch	MS Ny	MS 58	CS 58^{1st}	CS 58^{2nd}
St. 58	5.2	5.3	4.8	5.0	4.6	4.9	0	0	0	0
3A ♀ (16-20)	5.7	5.5	5.0	5.3	5.1	5.3	0	0	0	
$11AN_2$ (1-5)	6.2	6.1	5.6	6.1	>5.9	5.8	0	0	0	
21A ♀ (1-5)	4.8	4.8	4.3	4.4	4.3	4.3	0	0	0	
$8AN_1$ (31-35)	5.5	5.0	5.2	5.5	5.3	5.1	0	0	0	

VIRUS	NORMAL SERA		HETEROLOGOUS SERA						HOMOLOGOUS SERUM
	NMS	NCS	MS Qu	MS 60	CS 60^{1st}	MS Ny	MS 58	CS 58^{1st}	MS Ch
Chenuda	>8.3	>8.3	>8.3	>8.3	>8.3	>8.3	>8.3	>8.3	>5.3

NMS = Normal mouse serum
NCS = Normal chicken serum
MS Ny = Hyperimmune mouse serum against Nyamanini virus
MS 58 = Hyperimmune mouse serum against stock 2558-59
CS 58^{1st} = Immune chicken serum; first bleeding one month after immunizing inoculation
CS 58^{2nd} = Immune chicken serum; second bleeding one and half months after immunizing inoculation
MS Ch = Hyperimmune mouse serum against Chenuda virus
MS Qu = Hyperimmune mouse serum against Quaranfil virus
MS 60 = Hyperimmune mouse serum against stock 2560-61
CS 60^{1st} = Immune chicken serum; first bleeding one month after immunizing inoculation
CS 60^{2nd} = Immune chicken serum; second bleeding one and half months after immunizing inoculation

TABLE IV

ISOLATION OF QUARANFIL AND NYAMANINI VIRUSES
FROM FIELD-COLLECTED ARGAS ARBOREUS+

	1963																				1964			
	MAR		APR		MAY		JUNE		JULY		AUG		SEPT		OCT		NOV		DEC		JAN		FEB	
SEX Or STAGE	Quar.	Nyam.	Quar.	Nyam.	Quar.	Nyam.	Quar.	Nyam.	Quar.	Nyam.	Quar.	Nyam.	Quar.	Nyam.	Quar.	Nyam.	Quar.	Nyam.	Quar.	Nyam.	Quar.	Nyam.	Quar.	Nyam.
♂♂	1	3	0	1	0	0	0	2	0	4	0	0	1	0	1	3	2	1	1	2	0	3	2	3
♀♀	3	4	0	4	0	5	1	4	0	2	2	2	1	0	1	0	2	2	2	2	1	1	0	1
N_1N_1	3	0	2	1	2	0	0	1	1	2	0	1	0	1	0	4	1	0	1	1	2	0	1	1
N_2N_2	1	0	1	1	2	3	0	2	0	0	0	2	0	2	0	0	1	3	3	0	0	0	0	2
Virus Positive Pools *	8	7	3	7	4	8	1	9	1	8	2	5	2	3	2	7	6	6	7	5	3	4	3	7
No. Pools Tested	40		40		40		40		40		40		40		40		40		60		40		40	
Pool Inf. Rate %	20	17.5	7.5	17.5	10	20	2.5	22.5	2.5	20	5	12.5	5	7.5	5	17.5	15	15	11.7	8.3	7.5	10	7.5	17.5
Tick Inf. Rate % **	4.4	3.8	1.6	3.8	2.1	4.4	0.5	5.0	0.5	4.4	1.0	2.6	1.0	1.6	1.0	3.8	3.2	3.2	2.5	1.7	1.6	2.1	1.6	3.8
% Total Tick Inf. **	9.0		5.6		6.9		5.6		5.0		3.8		2.6		5.0		6.9		4.4		3.8		5.6	

\+ In every month except December, 1963, 10 pools of 5 ticks each were tested for each sex and nymphal stage

* Each pool consisted of 5 individual ticks ground and tested together

** Calculated

N_1= First nymphal instar plus a proportion of small individuals of the second nymphal instar

N_2= Second and third nymphal instars

TABLE V

VIRUS ISOLATIONS FROM ARGAS ARBOREUS ACCORDING TO SEX, STAGE, AND AMOUNT OF RESIDUAL BLOOD IN GUT AT TIME OF TESTING

SEX OR DEVELOP. STAGE	VIRUS	Virus Positive Pools According To: Am't Residual Blood Contained*			Sex and Stage		
		++++,+++	++	+,-	Quar.	Nyam.	Total
♂	Quar.	3	2	3	8 (19%)		30 (25%)
	Nyam.	9	2	11		22 (29%)	
♀	Quar.	5	2	6	13 (31%)		40 (34%)
	Nyam.	11	6	10		27 (35%)	
N_1	Quar.	7	5	1	13 (31%)		25 (21%)
	Nyam.	2	3	7		12 (16%)	
N_2	Quar.	1	4	3	8 (19%)		23 (20%)
	Nyam.	6	5	4		15 (20%)	
Total No. Virus Positive Pools		44	29	45	42 (100%)	76 (100%)	118 (100%)
No. Pools Tested		200	100	200	500		
% Pools Virus Positive		22%	29%	22.5%	8.4%	15.2%	23.6%

*Graded - (none) to ++++ (fullest) according to amount of residual blood present in specimens at time of testing for virus

TABLE VI

QUARANFIL VIRUS TRANSMISSION STUDY WITH
ARGAS ARBOREUS

TICK NO.	INITIAL INF. FEEDING: Day	INITIAL INF. FEEDING: Chick Fed Upon	INITIAL INF. FEEDING: $SMLD_{50}$* VIR/ML Ser.	REFEEDINGS: Day	REFEEDINGS: Feed Status	REFEEDINGS: Host Fed Upon	THE CHICK**: Day of Death	THE CHICK**: NT PFU /ML* 30 Day Ser.	THE TICK: Dead or Tested (Day)+	THE TICK: SM D/Inoc.	THE TICK: SM Survival (Days)	THE TICK: $SMLD^{*}_{50}$ Vir. /Tick.
Tr- ♀ 1	0	CH136	4.1	28	+?	Ch179		5.0	51			
Tr- ♂ 1	0	CH136	4.1	52	+++	Ch231		0	59	5/5	5	4.2
Tr- ♀ 2	0	CH136	4.1	35	+++	Ch207		5.2	51			
Tr- ♂ 2	0	CH136	4.1	28	+++	Ch180		4.3	52	6/6	5	4.2
Tr- ♀ 3	0	CH136	4.1						59	6/6	6	3.6
Tr- ♂ 3	0	CH136	4.1	28	+++	Ch181		5.0	59	6/6	6	
Tr- ♀ 4	0	CH136	4.1						28	6/6	6,7	
Tr- ♀ 5	0	CH136	4.1	28	+?	Ch182		4.7				
				52	++++	Ch233		0	87	0/6		
Tr- ♀ 6	0	CH138	5.0	29	++++	Ch183		0				
				53	++++	CH234	6					
				81	++++	H131		-	91	6/6	5	
Tr- ♂ 4	0	CH138	5.0	29	++	Ch183		0				
				53	++++	CH234	6		91	6/6	5	
Tr- ♀ 7	0	CH138	5.0						29	6/6	5	
Tr- ♂ 5	0	CH138	5.0						29	6/6	6	
Tr- ♂ 6	0	CH138	5.0	36	++	Ch210		5.0				
				53	++++	Ch235		0				
				85	++	Ch274			88	6/6	6,7	
Tr- ♀ 8	0	CH153	5.7	29	++++	Ch184		5.0	88	6/6	5	
Tr- ♀ 9	0	CH153	5.7						29	6/6	4	5.7
Tr- ♂ 7	0	CH154	5.0						60	6/6	6	
Tr- ♂ 8	0	CH154	5.0	36	+?	Ch214		5.5	52			
Tr- ♂ 9	0	CH154	5.0	53	+++	Ch240		4.0	65			
Tr- ♂ 10	0	CH154	5.0	36	+++	Ch216		5.0				
				60	++++	Ch253		0				
				95	+++	Ch280			95	6/6	6	3.1
Tr- ♀ 11	0	CH147	4.0	29	+?	Ch185		5.6				
				36	+?	Ch217		6.3				
				53	+++	CH241	6					
				83	++++	H131		-	93	6/6	5	4.5

* Expressed in log 10
** Following transmission refeedings
CH, ch = chick
CH = chick infected by tick bite
H131 = Immune heron which serum has neutralized 6.0 logs of virus in tissue culture

+ Ground singly in 1 ml of diluent and inoculated in suckling mice
→ "molted to" after initial feeding

TABLE VI (Cont.)

QUARANFIL VIRUS TRANSMISSION STUDY WITH
ARGAS ARBOREUS

TICK NO.	INITIAL INF. FEEDING			REFEEDINGS			THE CHICK**		THE TICK			
	Day	Chick Fed Upon	SMLD50* VIR/ML Ser.	Day	Feed Status	Host Fed Upon	Day of Death	NT PFU /ML* 30 Day Ser.	Dead or Tested (Day)+	SM D/Inoc.	SM Survival (Days)	SMLD*50 Vir. /Tick.
Tr- ♀ 12	0	CH162	5.7						23	6/6	5	
Tr- ♂ 12	0	CH162							23	6/6	5	
Tr- ♀ 13	0	CH162	5.7						23	6/6	4	
Tr- ♂ 13	0	CH162	5.7						23	6/6	5	3.7
Tr- ♀ 14	0	CH162	5.7	30	++++	CH219	5		47			
Tr- ♀ 15	0	CH163	4.5	23	++++	Ch187		0				
				47	++++	CH243	6					
				75	++++	H131		-	85	6/6	5,6	
Tr- ♂ 18	0	CH163	4.5	23	++++	Ch187		0				
				47	+++	CH243			85	6/6	5	
Tr- ♀ 16	0	CH163	4.5	23	++++	Ch188		5.5	43			
Tr- ♂ 15	0	CH163	4.5	23	++++	Ch188		5.5				
				43	+++	Ch244		0	54	6/6	5	
Tr- ♀ 17	0	CH163	4.5	23	++++	Ch189		5.2				
				47	++++	CH245	7		85	6/6	5	3.7
Tr- ♀ 18	0	CH163	4.5	23	+?	Ch190		5.0				
				30	+++	Ch220		5.5				
				54	+++	Ch254		0	82	6/6	5,6	
Tr- ♂ 17	0	CH164	5.5	30	++++	Ch221		-	46			
Tr- ♀ 19	0	CH167	4.3	23	++++	Ch191		5.4				
				47	++++	Ch246		0				
				75	++++	H131		-	85	6/6	5	
Tr- ♀ 20	0	CH168	5.5	23	+++	Ch192		5.2	46			
Tr- ♀ 21	Q	CH168	5.5	23	++++	Ch193		-				
				47	++++	CH247	7					
				75	++++	H131		-	85	6/6	5,6	
Tr- ♂ 19	0	CH168	5.5	75	++++	H131		-	85	6/6	6	
Tr- ♀ 22	0	CH168	5.5	23	++++	Ch194		0				
				47	++++	Ch248		0				
				79	++++	CH281	6		82	6/6	5	
Tr- ♀ 23	0	CH168	5.5	23	+++	Ch195		5.2				
				54	+++	Ch255		0				
				89	++++	Ch282			92	6/6	5	4.3
Tr- ♂ 20	0	CH172	3.3	29	+++	Ch222		5.0				
				47	+++	CH249	5					
				74	++++	H131		-	84	6/6	5	3.5
Tr-N_3→♀	0	CH138	5.0	53	++++	Ch250		0				
				85	++++	Ch275			88	6/6	6,7	3.3
Tr-N_3→♀	0	CH138	5.0	85	+++	Ch276			88	0/6		
Tr-N_3→♀	0	CH138	5.0	36	++++	Ch213		4.9	52			
Tr-N_2→N_3	0	CH153	5.7						30	6/6	5	4.7
Tr-N_1→N_2	0	CH164	5.5						23	6/6	5	3.4

TABLE VII

QUARANFIL VIRUS TRANSOVARIAL AND
TRANSSPERMATOPHORAL TRANSMISSION STUDY
WITH <u>ARGAS ARBOREUS</u>

Descent Level*	Parent Given Inf. Meal	Day Tested Following Inf. Meal of Parent	No. Tested	No. Infected
$1F_1LL$	♀ x ♂	23	5	0
	♀	23	5	0
	♀ x ♂	28	5	0
	♀	28	2	0
	♂	53	2	0
	♂	60	1	0
	$N_3 \rightarrow$ ♀	60	1	0
	$N_3 \rightarrow$ ♀	85	1	0
$2F_1LL$	♀ x ♂	47	4	0
	♀	47	2	0
	♀ x ♂	53	2	0
	♀	54	3	0
	♀	60	1	0
	♀	84	1	0
	♂	85	1	0
	♀ x ♂	87	1	0
	♂	95	2	0
$3F_1LL$	♀ x ♂	79	3	0
	♀	79	2	0
	♀ x ♂	85	1	0
	♀	89	1	0
	♀ x ♂	104	1	0
$4F_1LL$	♀ x ♂	96	2	0
	♀	97	1	0
	♀ x ♂	101	1	0
TOTAL			51	

*The F_1 larvae listed were from the first, second, third and fourth ovipositions respectively.

TABLE VIII

SUMMARY OF QUARANFIL VIRUS TRANSMISSION BY
ARGAS ARBOREUS

Extrinsic Incubation Period (Days)	23	28	29	30	36	43	47	52	53	54	60	79	85	89	95	Total
No. of Ticks Feeding	11	4	5	3	6	1	7	2	6	2	1	1	3	1	1	54
No. Fatally Infecting Host	0	0	0	1	0	0	5	0	3	0	0	1	0	0	0	10
No. Immunizing Host	7	4	3	1	6	0	0	0	1	0	0	0	0	0	0	22

TABLE IX

QUARANFIL VIRUS IN THE COXAL FLUID
OF ARGAS ARBOREUS

Extrinsic Incubation Period (Days)	18	19	24	25	44	45	50	51	Total
No. of Ticks Tested	1	9	1	6	1	6	4	4	32
No. With CX.FL. Infected	0	0	0	0	1	4	1	2	8

TABLE X

QUARANFIL VIRUS TRANSMISSION STUDY
WITH *ARGAS PERSICUS*

TICK DESIGNATIONS	INITIAL INF. MEAL			REFEEDINGS+		CHICK DEAD (DAY)	THE TICK**				
	DAY	CHICK FED UPON	SMLD$_{50}$* VIR/ML Ser.	DAY	CHICK FED UPON		NO., SEX OR STAGE	DAY TESTED	SM D/INOC.	SM SURVIVAL (DAYS)	SMLD$_{50}$ VIR. /TICK*
Tr- Stock 22 (23♂♂, 5♀♀)	0	CH143	5.7	47	CH223	5	1 ♂	89	6/6	6	3.4
				86	CH283	11	1 ♀	89	0/6		
							1 ♂	130	6/6	5	
							7 ♂♂	130	0/6		
							2 ♀♀	130	6/6	6	
							2 ♀♀	130	0/6		
Tr- Stock 42 (10♂♂, 1♀)	0	CH177	5.3	33	CH224	3✝	1 ♂	16	6/6	8	
							1 ♂	75	0/6		
							1 ♂	130	6/6	6	
							1 ♂	130	6/6	8	
Tr- Stock 43 (6♂♂)	0	CH178	5.0	33	Ch225	-	1 ♂	16	6/6	6	3.6
				72	Ch285	-	1 ♂	75	0/6		
							1 ♂	130	6/6	5	
Tr-♂ x ♀ 43a	0	CH178	5.0	33	Ch226	-	1 ♂	75	6/6	8	2.5
				72	Ch286	-	1 ♀	75	6/6	4	4.5
Tr- ♂ x ♀ 43b	0	CH178	5.0	33	Ch228	-	1 ♂	75	0/6		
				72	Ch287	-	1 ♀	75	6/6	6	
Tr- ♂ x ♀ 43c	0	CH178	5.0	33	Ch227	-	1 ♂	75	0/6		
				72	Ch288	-	1 ♀	75	6/6	6	
Tr- Stock 44 (15♂♂, 1N)	0	CH176	4.3	33	Ch229	-	1 ♂	16	1/6	7	
				72	Ch289	-	1 ♂	75	0/6		
							2 ♂♂	130	6/6	8	
							8 ♂♂	130	0/6		
Tr- ♂ x ♀ 44a	0	CH176	4.3	33	Ch230	-	1 ♂	75	0/6		
				72	Ch290	-	1 ♀	75	0/6		
Tr- N_2 → ♂	0	CH177	5.3				1 ♂	34	0/6		
Tr- N_2 → ♂	0	CH178	5.0				1 ♂	34	5/6	9	
Tr- N_2 → ♀	0	CH176	4.3				1 ♀	34	6/6	6	
Tr- N_2 → ♀	0	CH177	5.3				1 ♀	34	6/6	6	3.3
Tr- N_2 → N_3	0	CH177	5.3				1 N_3	34	1/6	8	
Tr- N_1 → N_2	0	CH143	5.7				1 N_2	89	6/6	6	3.3
Tr- N_1 → N_2	0	CH178	5.0				1 N_2	34	6/6	4	4.3

+ feeding status "++++"

✝ injured

* Expressed in log 10

** Ground singly in 1 ml of diluent and inoculated in suckling mice

CH = Chick infected by tick bite

TABLE XI

QUARANFIL VIRUS IN THE COXAL FLUID
OF *ARGAS PERSICUS*

Extrinsic Incubation Period (Days)	12	26	38	52	92	106	Total
No. of Ticks Tested	6	1	6	1	8	3	25
No. With CX FL Infected	0	0	0	0	1	0	1

TABLE XII

QUARANFIL VIRUS TRANSOVARIAL
TRANSMISSION ATTEMPTS WITH *ARGAS PERSICUS*

Descent Level*	Parent Given Inf. Meal	Day Tested Following Inf. Meal of Parent	No. Tested	No. Infected
$1F_1LL$	♀ x ♂	34	4	0
$2F_1LL$	♀ x ♂	72	3	0
	♀ x ♂	86	1	0
Total			8	

TABLE XIII

SLE VIRUS TRANSMISSION ATTEMPTS WITH
ARGAS ARBOREUS

Sex or Stage	Initial Inf. Meal* Day 0	Refeedings on Normal Chicks Day 24		Day 49		Ticks Tested		SM D/Inoc	SM Survival (Days)	$SMLD_{50}$ Vir/Tick (Log_{10})
	No. Fed	No. Fed**	No. Transmitting	No. Fed	No. Transmitting	Day	No.			
♂	6	3	0	4	0	52	6	0/6		
♀	11	9	0	9	0	24	1	0/6		
						52	1	6/6	6	5.5
							1	6/6	6	
							1	5/6	6	
							1	6/6	8	
							6	0/6		
$N_1 \rightarrow N_2$	1	-	-	-	-	24	1	6/6	7,8	
$N_2 \rightarrow$ ♀	1	-	-	1	0	52	1	0/6		
$N_2 \rightarrow$ ♂	2	-	-	-	-	24	2	0/6		
$N_3 \rightarrow$ ♀	3	-	-	1	0	24	1	0/6		
						52	2	0/6		
$N_3 \rightarrow$ ♂	2	-	-	-	-	52	2	0/6		

*Ticks fed on viremic chicks circulating 5.2 to 5.5 Log_{10} $SMLD_{50}$ of virus/ml of serum.

**Adults were fed in most instances on normal chicks in "infected" pairs (1 ♂ x 1 ♀ per chick); in 4 instances normal males were paired with infected females.

TABLE XIV

SLE VIRUS TRANSOVARIAL AND
TRANSPERMATOPHORAL TRANSMISSION STUDY
WITH ARGAS ARBOREUS

Descent Level*	Parent Given Inf. Meal	Day Tested Following Inf. Meal of Parent	No. Tested	No. Infected
$1F_1LL$	♀ x ♂	24	6	0
	♀	24	4	0
	♀	49	1	0
	$N_3 \rightarrow$ ♂	49	1	0
$2F_1LL$	♀ x ♂	49	6	0
	♀	49	2	0
TOTAL			20	

*The F_1 larvae listed were from the first and second ovipositions respectively.

APPENDIX

APPENDIX

REAGENTS AND WORKING SOLUTIONS

1. 25% Normal Rabbit Serum Diluent (25% NRS + A)

Normal rabbit serum	25.0 ml
Penicillin (100,000 u/ml)	1.0 ml
Streptomycin (40 mg/ml)	4.0 ml
Buffered water (pH 7.8)	70.0 ml
Yield	100.0 ml

2. H_2O + antibiotics (H_2O + A)

Normal rabbit serum in the above formula was replaced by buffered water (pH 7.8).

3. 10% Monkey Serum Saline plus Antibiotics (10% MSS + A)

Sterile saline	90.0 ml
Normal filtered monkey serum	10.0 ml
Mixture of 500 units of penicillin and 50 Ug of streptomycin per ml	1.6 ml

4. Borate Saline, pH 9.0

1.5 M NaCl	80.0 ml
0.5 M H_3BO_3	100.0 ml
1.0 N NaOH	24.0 ml
Distilled H_2O q.s. ad	1000.0 ml

5. Borate Saline, pH 9.3

1.5 M NaCl	80.0 ml
0.5 M H_3BO_3	100.0 ml
1.0 M NaOH	35.0 ml
Dist. H_2O	1000.0 ml

6. 0.4% Bovalbumin, Antigen-serum Diluent

4% bovalbumin pH 9.0	100.0 ml
Borate saline, pH 9.0	900.0 ml

7. Veronal Buffered Diluent (VBD)

Two solutions:

Sol. A.	NaCl	83.0 gm
	Sodium 5, 5-diethyl barbiturate	10.19 gm
	1 N Hydrochloric Acid	34.6 ml
	Stock solution containing 1M $MgCl_2$ and 0.3 M $CaCl_2$	5.0 ml
	Dist. H_2O q.s. ad	2000.0 ml
Sol. B	Gelatin	1.0 gm
	Dist. H_2O boiling	100.0 ml
	Dist. H_2O room temp	800.0 ml

One volume of Sol. A was added to 4 volumes of Sol. B. The pH of the mixture should be 7.3 to 7.4. VBD should be stored in the refrigerator not longer than 24 hours.

8. Hank's Balanced Salt Solution (HBSS; 10 X)

Sol. A.	NaCl	80.0 gm
	KCl	4.0 gm
	$MgSO_4 \cdot 7 H_2O$	1.0 gm
	MgCl . 6 H_2O	1.0 gm
	Dist. H_2O	400.0 ml
Sol. B.	$CaCl_2$	1.4 gm
	Dist. H_2O	60.0 ml
Sol. C.	Na_2HPO_4	0.6 gm
	KH_2PO_4	0.6 gm
	Phenol red solution (0.4%)	25.0 ml
	Dist. Water	500.0 ml

Solution A was slowly added to solution B with continuous stirring. Resulting mixture was added to solution C and the volume made up to 1000 ml with distilled water. Two ml of chloroform were then added as preservative and preparation stored at 4°C.

9. Lactalbumin Hydrolysate (LAH) Medium (2% Calf Serum)

Hanks BSS (10X)	98.0 ml
Dist. H_2O	768.0 ml
LAH (5% in dist. H_2O)	100.0 ml
Glucose (20%)	5.0 ml
$NaHCO_3$ (7.5%)	5.0 ml

Penicillin 2,000,000 units + Streptomycin

	1,000 mg + 20 ml dist. water ----------	2.0 ml
	Mycostatin ---------------------------	2.0 ml
	Calf serum (inactivated at 56°C for so min.) ---------------------------	20.0 ml

10. Earle's Balanced Salt Solution (10X)

Sol. A.	NaCl ----------------------------------	68.0 gm
	KCl -----------------------------------	4.0 gm
	$NaH_2PO_4 \cdot H_2O$ -------------------------	1.3 gm
	Dist. H_2) ----------------------------	800.0 ml
Sol. B.	$CaCl_2$ --------------------------------	2.0 gm
	$MgCl_2 \cdot 6\ H_2O$ -------------------------	1.7 gm
	Dist. H_2O ----------------------------	100.0 ml

Solution B was added to solution A and the volume adjusted to 1 liter then autoclaved at 115°C for 10 minutes.

11. Agar Overlay Medium (1% Noble Agar).

Sol. A.	Earle's BSS (10X) --------------------	49.0 ml
	Dist. H_2O ----------------------------	150.0 ml
	Yeast-LAH extract solution (see.12) ---	16.5 ml
	New born calf serum ------------------	10.0 ml
	Glucose ------------------------------	2.5 ml
	$NaHCO_3$ (7.5%) ------------------------	15.0 ml
	Pen. & Strep. (see 9) ----------------	1.0 ml
	Mycostatin ----------------------------	1.0 ml
	Neutral red solution (1:1000) ---------	8.3 ml

Sol. B. Nobel agar (Difco) -------------------- 5.0 gm

Dist. H_2O ---------------------------- 250.0 ml

Solution B was autoclaved at 115°C. for 10 minutes, dispensed in 100 ml amounts and stored at 56°C. Solution A and Solution B were combined in equal parts immediately before use.

12. Yeast Extract - Lactalbumin Hydrolysate Solution

Sol. A. Yeast extract powder ------------------ 2.0 gm

Dist. H_2O at 56°C. -------------------- 98.0 ml

Sol. B. LAH ---------------------------------- 10.0 gm

Dist. H_2O at 80°C. -------------------- 90.0 ml

The two solutions were cooled then combined. After filtration through a millipore filter the mixture was stored at 4°C.